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4) Int. J. Obesity, 2000, Vol. 24, pp. 989-996.

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LETTER

Increased adiposity due to viral infection in animals

Dhurandhar *et al*, in a recent paper in this journal,[1] suggest a causal link between a viral infection and an increase in adiposity[1] in experimental animals. However, their otherwise meticulous work suffers from a lack of rigor in statistical analysis and interpretation of the results. The main flaws, in our opinion, affect the interpretation of experiment 1, the analysis of experiment 4 and the comparison of prevalence of obesity across groups.

In experiment 1, two groups, infected respectively by human Adenovirus-36 (Ad-36) and an avian adenovirus type (CELO), were compared to a control group. A significant difference in visceral and total body fat was found between the Ad-36 and the control group. While the CELO group exhibited intermediate values for body weight and visceral fat, it did not differ significantly from the control group. From the data shown, it can easily be verified that neither did it differ significantly from the Ad-36 group. Given this, and the low statistical power of the experiment (only 13 animals per group), the conclusion that 'the first experiment demonstrated that CELO [...] did not produce adiposity similar to that produced by Ad-36 [...]' appears inappropriate.

In experiment 4, rejecting an animal from the control group when analysing the data is highly questionable in a randomized weight-matched experiment, particularly if selection is based solely on the outcome variables being analysed. From the data provided, it is possible to compute what would have been the mean and standard deviation for body weight in the control group had that animal not been withdrawn (mean = 28.99 g and s.d. = 3.96). The *z*-score for the 'outlier' value, 3.38 g, was thus 2.48, which corresponds to the 0.99 probability level of the Gaussian cumulative distribution function. We can, therefore, deduce that in a sample of 10 animals the likelihood of having at least one value above or equal this level is $1 - 0.99^{10} \approx 0.10$. It is also noteworthy that the above standard deviation is much closer to the one in the inoculated group (4.25) than the post-rejection one (2.37), which differs significantly from the latter ($F_8^{19} = 3.22$, $P < 0.05$). The same pattern of results holds for visceral fat. The likeliest conclusion of these observations is that, rather than eliminating an outlier, the authors did cut off the upper tail of the distribution in the control group. Strictly speaking, then, no significative difference for weight and visceral fat can be found between both groups (we lack the necessary data to recompute tests for other variables). Equal sample sizes, rather than the chosen 10 *vs* 20 scheme, would have reduced the influence of an extreme observation in the control group and yielded optimal statistical power.

The method chosen to compare the prevalence of obesity is statistically incorrect. Since the prevalence of obesity in the control group was fixed by the investigator, it was not a random variable for this group. Furthermore, since the prevalence in the Ad-36 group depends on the data in control group, the two groups cannot be considered independent for this variable. Hence the Chi-square test is invalid. It would also be interesting to know what algorithm was used to compute the 85th percentile in a sample of eight animals, and what were the confidence intervals of such estimates. Even if they were correct, these tests would not add further information since they would just be another (less efficient) way to compare visceral adiposity.

Finally, extreme caution should be exerted in drawing parallels between what may be a mere acute infection in animals and a chronic disease state in humans. The chickens were followed up for only a short time after inoculation. After 13 weeks (experiment 3), the difference in visceral fat, expressed as a percentage of the control group mean, was lower. However, different doses and routes of infection preclude direct comparisons between different durations of experiment. Since no significant weight difference was demonstrated, it would have been preferable not to let the word obesity[2,3] appear in this paper,[1] in spite of the fascinating questions it raises...

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REPLY

We would like to thank Drs Preziosi and Czernichow for their critical evaluation of our paper, recently published in the *International Journal of Obesity*.[1] The main concerns of Preziosi and Czernichow were with some of the statistical approaches that we used to draw conclusions. Before we explain our approach, we would like to assure them that we have replicated these results in several experiments. Four of these experiments were combined and reported in this paper. Undoubtedly, these preliminary results should be replicated in a larger number of animals, by us and other researchers, to make a stronger case for Ad-36-induced adiposity in animals. We agree that study designs could be changed or improved to address or confirm the questions raised in this study.

We do not agree that the conclusion drawn in experiment 1 is inappropriate due to low statistical power. Although adding more animals would have increased the power in experiment 1, Allison *et al*[2] have shown that in unbalanced groups with a subject ratio of 2:1 the resulting reduction in power is not dramatically lower. Also, the fact that statistically significant differences were obtained despite the small numbers is, in our opinion, more impressive, particularly as we used the stringent Bonferroni adjustment following the *t*-test for this experiment. In addition, one-way ANOVA of the control, CELO and Ad-36 groups showed $P=0.01$ for visceral fat but $P=0.30$ for body weight, and further supports our conclusions. Thus, although a low statistical power could be a concern before conducting an experiment, it is much less relevant when an experiment has already yielded significant differences.

We disagree with the comment about the treatment of the outlier in experiment 4. Although there are several approaches to the treatment of outliers in performing data analysis, we have used a perfectly acceptable approach of excluding the data point if it qualifies as an outlier. As stated in the paper, the outlier animal had nearly 40% greater body weight and 305% greater visceral fat compared to the respective means of the rest of the control group. This animal qualified as an outlier by two seprate statistical tests (Q Distribution Deviate and Maximum Normal Residual Test), and was therefore excluded from the analysis. As stated in the paper, this was a preliminary experiment to investigate the adipogenic effect of Ad-36 in a mouse model, and we agree that equal sample sizes may be a better design for future studies.

We agree that tests better than 2×2 Chi square could have been used to assess the significant differences in adiposity-related variables in the four experiments. In fact, this categorical analysis was only meant to supplement our more formal analyses comparing the group means on continuous dependent variables, such as fat mass. In this context, it should be noted that the title of the paper states that adiposity (not obesity) is increased in the virus-infected animals.

We strongly disagree about the usefulness and the appropriateness of the prevalence of obesity that was calculated. The Centers for Disease Control have used the 85th percentile of body weight as the cut-off point to define obesity in the NHANES surveys in the United States. Obviously, there are no definitions for obesity in animals. Human definitions of obesity use body weight and/or body mass index (BMI) for convenience and as surrogate measures for increased body fat. Many obesity researchers would prefer to define human obesity based on body fat content.[3] Therefore, defining and calculating the prevalence of virus-induced obesity in animals is neither inefficient nor irrelevant to human obesity. The 85th percentile was determined arithmetically, using Microscoft Excel.

Thirteen weeks and 21 weeks represent significant portions of the life spans of chickens and mice, respectively. Therefore, we do not agree that these were short-term studies. We do not understand the relevance of the comment about different doses and routes of infections precluding direct comparisons. Our paper clearly stated the purpose of each of the four experiments, and each stands independently. Also, in contrast to the statement by Drs Preziosi and Czernichow, the percentage visceral fat in experiment 3 is very similar to (not lower than) that obtained in experiment 2. It appears that they may have compared the visceral fat of chickens with that of the mice in experiment 4.

In humans, body weight is used as a convenient operational marker to determine the degree of obesity, although it is strictly an excessive accumulation of body fat and not weight *per se*. Therefore, body fat is a more relevant determinant of obesity than body weight, and in the paper we refer to the 'increased adiposity' due to Ad-36. As stated in the paper, 'obesity was only arbitrarily defined in order to express the response of individual animals to Ad-36 infection.

In summary, these studies should be considered as a beginning and not an end, and the suggestions of Drs Preziosi and Czernichow should prove useful when conducting future studies.

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Human Adenovirus Ad-36 Promotes Weight Gain in Male Rhesus and Marmoset Monkeys[1,2]

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ABSTRACT Although obesity has multiple etiologies, an overlooked possibility is an infectious origin. We previously identified two viruses, SMAM-1, an avian adenovirus (Ad), and Ad-36, a human adenovirus, that produce a syndrome of visceral obesity, with paradoxically decreased serum cholesterol and triglycerides in chickens and mice. In the two studies presented in this paper, we used nonhuman primates to investigate the adiposity-promoting potential of Ad-36. In study 1, we observed spontaneously occurring Ad-36 antibodies in 15 male rhesus monkeys, and a significant longitudinal association of positive antibody status with weight gain and plasma cholesterol lowering during the 18 mo after viral antibody appearance. In study 2, which was a randomized controlled experiment, three male marmosets inoculated with Ad-36 had a threefold body weight gain, a greater fat gain and lower serum cholesterol relative to baseline ($P < 0.05$) than three uninfected controls at 28 wk postinoculation. These studies illustrate that the adiposity-promoting effect of Ad-36 occurs in two nonhuman primate species and demonstrates the usefulness of nonhuman primates for further evaluation of Ad-36–induced adiposity. J. Nutr. 132: 3155–3160, 2002.

KEY WORDS: • *cholesterol* • *adiposity* • *obesity* • *nonhuman primates* • *infection*

Obesity has multiple etiologies, but infectious agents have been consistently overlooked as a possible origin of human obesity. Three animal viruses have been reported to cause obesity in nonprimate species, but have not been implicated in initiating or maintaining obesity in humans (1–3). We have now identified two additional viruses, SMAM-1, an avian adenovirus (Ad), and Ad-36, a human adenovirus, that produce obesity in animals (4–7). In 6 separate experiments, we have shown that these two adenoviruses produce a syndrome of visceral obesity, along with paradoxically decreased serum cholesterol and triglycerides in chickens and mice (4–8). A capillary electrophoresis assay designed to detect Ad-36 DNA (9) showed tropism of the virus for adipose tissue of the infected animals (6,7). Ad-36–induced adiposity in animals was hyperplastic and hypertrophic (10). Preliminary data showed a marked up-regulation of adipocyte differentiation induced by Ad-36 (11,12). In humans, serum antibodies to both SMAM-1 and to Ad-36 are associated with obesity and lower cholesterol and triglycerides levels (5,13). For ethical reasons, the definitive experiment of injecting humans with Ad-36 to determine a causal role for this virus in human obesity is not possible. Nonhuman primates are the best surrogates for human experiments. In this paper, we used two disparate nonhuman primate species as models in which to study the adiposity promoting potential of Ad-36.

MATERIALS AND METHODS

Study 1: spontaneously occurring antibodies to Ad-36 in rhesus monkeys. Adult male rhesus monkeys (*Macaca mulatta*) were screened for the presence of spontaneously occurring antibodies to Ad-36, to ascertain their association with longitudinal changes in body weight and cholesterol. Frozen plasma samples from adult male rhesus monkeys ($n = 15$) were obtained from the Wisconsin Regional

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TABLE 1

Composition of diet offered to rhesus monkeys[1,2]

	unit/kg
Energy, kJ (kcal)	16,828 (4020)
Protein, g	150
Crude fat, g	100
Crude fiber, g	50

[1] Minerals: contains calcium, 7.9 g; phosphorus, 4.9 g; potassium, 3.6 g; magnesium, 1.2 g; sodium, 2.1 g; chloride 3.2 g; iodine, 2 mg; copper 10 mg; selenium, 1 mg; chromium, 2 mg; iron, 276 mg; zinc, 51 mg; manganese, 62 mg.

[2] Vitamins: contains riboflavin, 22 mg; niacin, 99 mg; pantothenic acid, 55 mg; folic acid, 2 mg; thiamin, 19 mg; biotin, 4 mg; choline, 1259 mg; cholicalciferol, 34 mg; vitamin A, 40 mg; vitamin K, 50 mg; vitamin E, 227 mg; vitamin B-12, 30 μg; vitamin C, 1017 mg.

Primate Research Center (WRPRC),[4] Madison, WI. Blood samples and body weight measurements were collected every 6 mo for 90 mo. Monkeys were offered once daily a purified diet (#85387, Teklad, Madison, WI) supplemented daily with a piece of fresh fruit. The composition of the diet is described in **Table 1**. Energy value of the diet eaten was ~167 kJ [700 kcal/(monkey · d)]. Monkeys consumed water ad libitum. All monkeys were between 8 and 14 y of age when the first plasma sample available for this study was drawn (baseline sample). Plasma samples were used to determine total cholesterol as well as antibodies to Ad-36, using the neutralization assay described below. For each monkey, the time of first appearance of Ad-36 antibodies, as well as body weight and cholesterol were determined for 18 mo before and after the first antibody appearance.

Study 2: infection of marmosets with Ad-36. In a randomized experiment, adult male common marmosets (*Callithrix jacchus*) were inoculated with Ad-36 to investigate prospectively the adiposity-promoting effect of the virus. Ad-36 antibody-free adult male marmosets (n = 6, age range 2–6 y) were obtained from the WRPRC and were divided into two weight- and age-matched groups (each n = 3) that were individually housed in two separate rooms. Marmosets were fed once daily at 1230–1400 h ~20 g of a specialized diet (**Table 2** Zu Preem Marmoset Diet, Premium Nutritional Products Topeka, KS). Two small chunks of fruit (varied daily, ~10 g) were added to provide variety and ~5 mL of yogurt was spread on top, providing supplements of vitamin C, cholecalciferol, and calcium. Based on our previous experience, 20 g of the diet is slightly more that the amount consumed by marmosets in a day. To verify ad libitum consumption, it was noted that some food remained in the cage each day. Monkeys consumed water ad libitum. After 4 wk of acclimatization, marmosets were anesthetized with ketamine-xylazine [10 and 0.5 mg/kg, respectively, intramuscular (i.m.)] and inoculated intranasally (i.n.) with tissue culture media containing either Ad-36 virus (5×10^5 plaque forming units; Ad-36 group) or no virus (control group). Blood samples were drawn before inoculation (baseline) and also 10, 17 and 28 wk postinoculation and used for antibody screening (using the serum neutralization method described below) and for determination of serum cholesterol and triglycerides. Using the stable isotope dilution method described below, total body fat was determined at baseline and at 28 wk postinoculation, when the experiment was terminated and the monkeys were killed by administration of an i.m. injection of 10 mg/kg ketamine and 0.5mg/kg xylazine followed by an intravenous (i.v.) injection of 100 mg/kg pentobarbital. Visceral fat (intraperitoneal fat) was carefully removed from each carcass and weighed. Samples (~1 g each) of the visceral fat, liver, skeletal muscle, lung and brain of all monkeys were flash-frozen in liquid nitrogen to screen for Ad-36 DNA using a nested polymerase chain reaction (PCR) assay.

[4] Abbreviations used: Ad, adenovirus; ATCC, American Type Culture Collection; CPE, cytopathic effect; IACUC, Institutional Animal Care and Use Committee; i.m., intramuscular; i.n., intranasal; I.v., Intravenous; MEM, minimum essential media Eagle; PCR, polymerase chain reaction; WRPRC, Wisconsin Regional Primate Research Center.

Animal care. Rhesus and marmoset procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Wisconsin at Madison. Monkeys were housed individually in cages that allowed auditory, visual and olfactory contact. The Ad-36 group and the uninfected control marmosets were housed in two separate rooms under biosafety level 2 containment.

Tissue culture techniques. A549 cells (human lung carcinoma cells) obtained from American Type Culture Collection (ATCC, Manassas, VA) were used to grow Ad-36. Minimum essential medium Eagle (MEM) (Cat # M-0643, Sigma Chemical, St. Louis, MO) with nonessential amino acids, Earle's salts, L-glutamine, 10% fetal bovine serum and 2.9% $NaHCO_3$ (v/v), pH 7.4, was used for growing the cells.

Virus growth. Ad-36 was obtained from the ATCC. The virus was plaque purified as previously described (6,7) and grown in A549 cells. A working stock of virus was prepared as previously described (6,7) and was titrated on A549 cells, divided into aliquots and stored at −80°C.

Serum neutralization test for detecting antibodies. Rhesus plasma and marmoset serum were tested for the presence of Ad-36 antibodies. The assay used A549 cells and was conducted as a constant-virus-decreasing-serum method, as previously described (6,7). The absence of cytopathic effect (CPE) of the virus on A549 cells in the presence of the test serum is considered an indication of effective neutralization of the virus and the serum is considered to have antibodies against the virus. Samples were considered antibody positive if the serum titer was ≥1:8. A few rhesus samples demonstrated cell-toxicity up to 1:16 dilutions. For these samples, a more stringent criterion of titer (≥1:32) was used to denote antibody positivity.

Cholesterol assay. Fasting total cholesterol was determined in duplicate with a cholesterol-oxidase-peroxidase method (Cat # 352–500P; Sigma Chemical) using 10 μL of serum.

Development of a nested PCR assay for detection of Ad-36 DNA. Four primers were designed to unique regions of the Ad-36 fiber protein gene for use in nested PCR detection of viral DNA. Sequences of primers were as follows: outer forward primer (5′-GTCTGGAAAACTGAGTGTGGATA), outer reverse primer (5′-ATCCAAAATCAAATGTAATAGAGT), inner forward primer (5′-TTAACTGGAAAAGGAATAGGTA), inner reverse primer (5′- GGTGTTGTTGGTTGGCTTAGGATA). DNA was isolated using a QIAamp Tissue Kit (Cat #29304; Qiagen, Valencia, CA). Negative PCR controls were water and DNA from uninfected A-549 cells. Positive PCR control was DNA from Ad-36 infected A-549 cells. DNA was denatured for 2 min at 95°C and subjected to 35 cycles of PCR (94°C for 1 min, 55°C for 1 min, 72°C for 2 min) followed by incubation at 72°C for 5 min. PCR products were visualized on a 1% agarose gel with a size marker. Nested PCR products from positive control (Ad-36 DNA) and infected marmoset brain tissue were sequenced to confirm amplification of targeted region of the gene.

Virus isolation. Adenovirus infection in marmosets was confirmed by collecting fecal samples (wk 4 and 9 after Ad-36 inoculation) and growing the virus on cell cultures as described earlier (7).

TABLE 2

Composition of the canned diet offered to marmosets[1,2]

	unit/kg
Energy, kJ	6,907 (4020)
Protein, g	93.3
Fat, g	32
Ash, g	24
Fiber, g	2.5

[1] Minerals: contains calcium, 3.3 g; phosphorus, 2.4 g; sodium, 2.2 g; potassium, 3.3 g; magnesium, 0.5 g; iron, 54 mg; zinc, 56 mg; copper, 5.3 mg; iodine, 0.8 mg; manganese, 7.4 mg.

[2] Vitamins: contains vitamin A, 42 mg; cholicalciferol, 0.235 mg; vitamin E, 94 mg; thiamine, 40.0 mg; riboflavin, 8.0 mg; pyridoxine, 5.2 mg; niacin, 55.0 mg; pantothenic acid, 25.0 mg; biotin, 0.28 mg; folic acid, 0.2 mg; choline, 587 mg.

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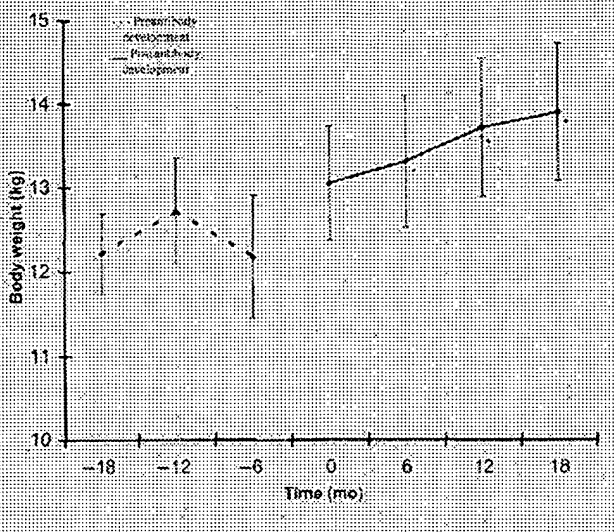

FIGURE 1 Adenovirus (Ad)-36 antibody appearance and body weight change in rhesus monkeys. Values are mean ± SD; $n = 8$. Plasma samples from adult male rhesus monkeys were collected every 6 mo for 90 mo and tested for antibodies to Ad-36. Body weights are plotted for the 18 mo before the onset of Ad-36 antibodies (−18, −12 and −6 mo) and after the monkeys became antibody positive (0, 6, 12 and 18 mo). *Different from the body weight at −6 mo (the last antibody-free value), $P < 0.05$.

Body composition analysis by stable isotope dilution method. Blood (1 mL) was drawn followed by i.v. administration of 0.1 g/kg deuterium oxide. The dose was determined by weighing the syringe before and after administration. A second blood sample was obtained 2 h after the dose. Equilibration time for the stable isotope is ~60–75 min for rhesus monkeys; therefore, 2 h was considered an adequate equilibration period for the marmosets. Isotope dilution spaces were determined by forcing the serum through a 100,000-Da exclusion filter and distilling and reducing over zinc for mass spectrometric determination of the isotopic abundance. Dilution space was calculated from the isotopic enrichment of the 2-h sample relative to the predose sample. Fat-free mass was calculated from total body water, assuming a hydration ratio of 0.732. Body fat was calculated by subtracting fat-free mass from the total body mass.

Statistical analysis

Study 1. *Multivariate (O'Brien) test.* Before conducting univariate tests and to increase power, a multivariate test, in which the dependent variable Y was defined as $Y = (2\text{Weight} - 2\text{Cholesterol})$ was used (14). When Y was regressed on Ad-36 status, monkey, and the polynomials of age in the mixed effects model, the effect of Ad-36 status was significant ($F = 8.14$; df = 1,96; $P = 0.005$). Thus, there was a clear effect of Ad-36 status on combination of weight and cholesterol. We then examined each dependent variable separately.

Univariate tests. Univariate tests were conducted only when the multivariate test was significant. Each dependent variable (weight and cholesterol) was regressed on Ad-36 antibody status, monkey, and polynomials of age in a mixed-effect model.

Study 2. Groups were compared using Student's *t* test. Values in the text are means ± SD.

RESULTS

Study 1: spontaneously occurring antibodies to Ad-36 in rhesus monkeys. During the 90-mo period, all 15 monkeys showed Ad-36 antibodies at some point in time. Because blood samples were obtained at 6-mo intervals, the exact date of seroconversion during the preceding 6 mo of the first positive sample is unknown. The appearance of antibodies in the plasma did not demonstrate an annual pattern. Of 15 monkeys, 8 were seronegative at baseline. Only these monkeys were included in the statistical analysis to compare the body weights and cholesterol levels before and after the first appearance of Ad-36 antibodies. The remaining 7 monkeys were seropositive for Ad-36 antibodies at baseline and were excluded from statistical analysis.

Before separate univariate tests were conducted, a multivariate test (see Methods) was conducted that indicated a clear effect of Ad-36 status on the combination of weight and cholesterol ($P = 0.005$).

The monkeys were completely free of antibodies from −18 to −6 mo before the first positive sample; this was designated as the "baseline" period, whereas the period from +6 to +18 mo after seroconversion was designated as the "postinfection period." Body weight and plasma cholesterol level changes for 18 mo before and 18 mo after the first appearance of Ad-36 antibody are presented in Figures 1 and 2. Time point "0 mo" denotes the first antibody positive serum sample for each monkey.

Weight. Body weight changed little during the baseline period, decreasing by −0.3% (0.04 ± 1.5 kg; $P = 0.87$). In contrast, the body weight increased by ~10% in 6 mo and by ~15% (1.7 ± 0.8 kg; $P < 0.03$) at 18 mo during the "postinfection" period (Fig. 1). In the full statistical model, the effect of Ad-36 antibody status was significantly associated with weight gain ($F = 5.47$; df = 1,96; $P = 0.021$). The parameter estimate for the effect of Ad-36 antibody status was 0.81 kg, indicating that the generation of Ad-36 antibodies was associated with an increase in body weight of 0.81 kg.

Cholesterol. Plasma cholesterol levels were stable during the baseline period, but decreased by −23% ($P = 0.06$) during the 6 mo immediately after the appearance of Ad-36 antibodies and remained low for at least 18 mo during the postinfection period (Fig. 2). In the full statistical model, the effect of Ad-36 antibody status was significant ($F = 5.33$; df = 1,96; $P = 0.023$). The parameter estimate for the effect of Ad-36 antibody status was 0.49 mmol/L, indicating that the generation of Ad-36 antibodies was associated with a decrease in

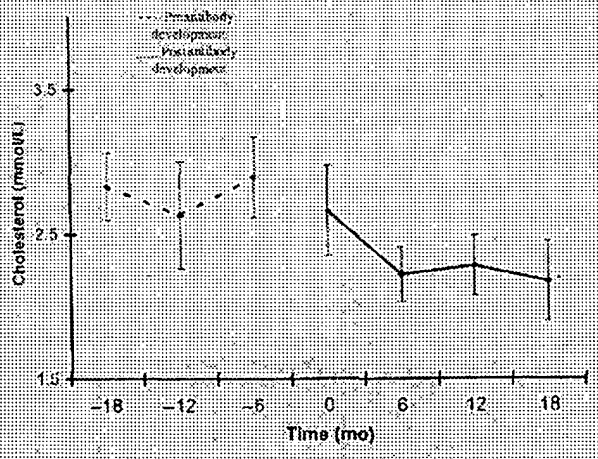

FIGURE 2 Adenovirus (Ad)-36 antibody appearance and plasma cholesterol change in rhesus monkeys. Values are mean ± SD; $n = 8$. Plasma samples from adult male rhesus monkeys were collected every 6 mo for 90 mo and tested for antibodies to Ad-36. Plasma cholesterol values are plotted for the 18 mo before the onset of Ad-36 antibodies (−18, −12 and −6 mo) and after the monkeys became antibody positive (0, 6, 12 and 18 mo). *Different from the body weight at −6 mo (the last antibody-free value), $P < 0.05$.

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plasma levels of cholesterol by 0.49 mmol/L. Thus, when controlled for age, and after the first appearance of Ad-36 antibody, the rhesus monkeys had greater body weights and lower cholesterol levels.

Study 2: infection of marmosets with Ad-36. Body weights of both groups did not differ at the time of inoculation (336.4 ± 19.8 g vs. 346.3 ± 27.8 g, $P = 0.65$, for the Control and the Ad-36–infected groups, respectively). The serum neutralization assay showed an absence of Ad-36 antibodies in the control group at all times during the experiment. Two of the three Ad-36–inoculated marmosets were antibody positive at both 10 and 17 wk postinoculation. Ad-36 could not be isolated from the fecal samples of the control group at any time during the study. However, the infective virus was isolated at both 4 and 9 wk postinoculation from the fecal samples of the two Ad-36 inoculated marmosets that had detectable Ad-36 antibodies. The nested PCR assay, however, detected Ad-36 DNA in the adipose tissue, liver, skeletal muscle, lung and the brain samples of all three Ad-36 inoculated marmosets, but not in any monkey in the control group. The nested PCR assay results for the adipose tissue of the two groups are presented in Figure 3. Isolation of Ad-36 virus from fecal samples and the presence of viral DNA in the tissue of all infected males demonstrated that marmosets were readily infected with Ad-36.

At 28 wk after inoculation, the Ad-36 group had greater body weight gain (41.4 ± 11.2 g vs. 10.8 ± 13.4 g, $P = 0.039$; Fig. 4) and total body fat gain (36.3 ± 6.1 g vs. 23.0 ± 3.0 g, $P = 0.013$) than controls. The mean weight gain of 41 g represented a 12% increase in the Ad-36 group compared with a 3.2% increase in the control group, a threefold difference. The Ad-36 group tended to have more visceral fat than controls (7.3 ± 2.5 g vs. 4.4 ± 0.9 g, $P = 0.089$). Despite the gain in fat mass and body weight, serum cholesterol levels were lower in the Ad-36 group than in controls (change from baseline, −0.79 ± 0.28 mmol/L vs. 0.10 ± 0.08 mmol/L, $P = 0.006$; Fig. 5). The change in serum triglycerides from baseline tended to be greater in infected marmosets that in controls (−0.52 ± 0.23 mmol/L vs. 0.14 ± 0.53 mmol/L, $P = 0.122$).

DISCUSSION

Although 50 types of human adenoviruses are deposited with the ATCC, Ad-36 is the first reported to cause obesity in animals (6,7). Ad-36 is serologically different from at least 47 of the other 49 human adenoviruses (15–22); it was first isolated in 1978 in Germany from the feces of a diabetic girl suffering from enteritis (17). Relative antigenic uniqueness was one of the main reasons for selecting Ad-36 to test for the adiposity-promoting effect.

The concept of virus-induced obesity is of greater importance if shown to be relevant to human obesity. Although we have demonstrated the adipogenic and hypolipidemic effects of a human virus in animals such as chickens and mice, we can not conclusively extrapolate the results, without verification, to human obesity. Differences in lipid metabolism between lower animals and primates preclude such a direct extrapolation. For instance, the energy metabolism of chickens is based on free fatty acids, not glucose, and insulin is of minor importance. Such metabolic differences clearly warranted the use of a higher model to establish the relevance of the findings to human obesity. In addition, because of ethical reasons, humans can not be infected experimentally with Ad-36 to verify its adipogenic effect directly. Therefore, we decided to use nonhuman primate species as the best way of determining the relevance of Ad-36 in human obesity.

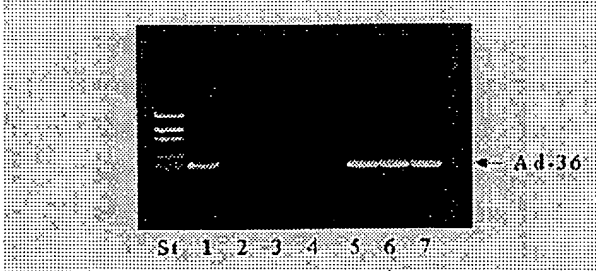

FIGURE 3 Nested polymerase chain reaction assay for adenovirus (Ad)-36 DNA detection in the marmoset adipose tissue ($n = 6$). Key: St. DNA ladder, 1: Ad-36 DNA positive control, 2–4: adipose tissue DNA from the uninfected control marmosets, 5–7: adipose tissue DNA from the Ad-36 infected marmosets.

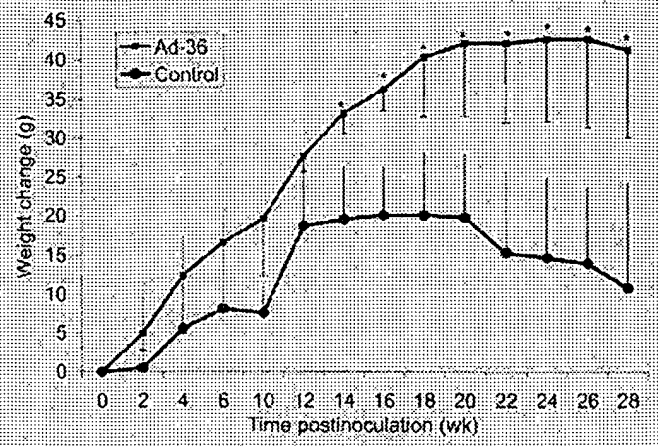

FIGURE 4 Cumulative body weight gains in marmosets after inoculation with adenovirus (Ad)-36. Values are means ± SD, $n = 3$ per group. *Different from control, $P < 0.05$.

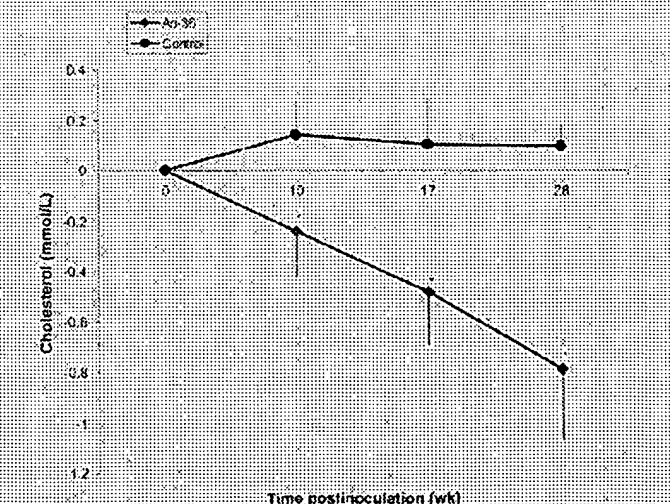

FIGURE 5 Cumulative changes in serum cholesterol in marmosets after inoculation with Ad-36. Values are means ± SD, $n = 3$ per group. *Different from control, $P < 0.05$.

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In our rhesus monkey study, the fact that all of the 15 monkeys had Ad-36 antibodies at some time during the 90-mo period suggests a covert epidemic of Ad-36 infection in the rhesus monkey colony at WRPRC. The source of Ad-36 infection in monkeys is not yet known. However, transmission from humans is a possibility. Although most adenoviruses are species specific in their replication cycle, we have recovered infectious Ad-36 virus from Ad-36–infected chickens (6,7) and have detected naturally occurring antibodies to Ad-36 in chickens as well as rats (unpublished data). The ability of Ad-36 to infect and replicate in widely disparate vertebrate species is in itself unusual. Therefore, the presence of naturally occurring antibodies in rhesus monkeys to a human adenovirus is not very surprising.

Serum neutralization is considered the "gold standard" to specifically detect neutralizing antibodies. In addition to the previously reported minimal antigenic cross-reactivity of Ad-36 (16,–22), we confirmed that Ad-36 does not cross-react with other human adenoviruses such as Ad-2, Ad-31 or Ad-37 (unpublished data). Therefore, it is unlikely that the antibodies detected in the rhesus monkey plasma that neutralized Ad-36 were antibodies to other human adenoviruses. Antibodies to simian adenovirus(es) that may cross-react with Ad-36 are a possibility, although cross-reactivity between a simian adenovirus and Ad-36 has not been reported.

In rhesus monkeys, a positive correlation of age with body weight and serum cholesterol would have been expected. The increase in body weight and drop in cholesterol during the postinfection period that persisted even after controlling for age supports our hypothesis that these changes were associated with the appearance of Ad-36 antibodies.

A comparison of body weight and plasma cholesterol changes between antibody positive and antibody-free monkeys would have been optimal to eliminate the effect of age on these variables. However, all 15 rhesus monkeys developed Ad-36 antibodies at different times during the study period, thus precluding such a comparison. Nevertheless, the following measures ensure that the changes in body weight and cholesterol observed were not age related.

The age of the monkeys ranged from 8 to 14 y. The age of the first appearance of antibodies varied among the monkeys. Regardless of the age of seroconversion, there was little change in body weights or plasma cholesterol in the 18 mo before the first appearance of antibodies. Thus, although the period before the onset of antibodies represents different ages for individual monkeys, their body weights were stable during this period. Such a stabilization of body weight is expected in adult rhesus monkeys who have completed their growth. Also, an age-related decline in plasma cholesterol is not expected in freely-fed rhesus monkeys. In effect, each monkey acted as his own control for body weight and cholesterol comparisons before and after the seroconversion. As stated earlier, we further ascertained the age effect by controlling statistically for age, and found a clear effect of seroconversion on body weight and cholesterol that was independent of age. These measures demonstrate that the observed changes were not age related.

In the marmoset experiment, the persistence of Ad-36 DNA in various tissues of all marmosets 6 mo postinfection is an important finding, demonstrating the spread of Ad-36 in their bodies, even though one marmoset failed to generate detectable levels of antibodies. In addition, the presence of Ad-36 DNA in the adipose tissue is particularly intriguing because our preliminary data showed Ad-36 induced up-regulation of 3T3-L1 cell (rodent embryonic preadipocytes) differentiation (11,12). Future experiments should investigate the Ad-36–induced up-regulation of fat cell differentiation as a possible mechanism of Ad-36–induced obesity in monkeys. Similarly, Ad-36–induced hypothalamic damage is another possible mechanism that should be investigated. Canine distemper virus may promote obesity in mice by inducing hypothalamic damage (1). Although we did not find histopathological hypothalamic lesions in Ad-36–infected mice in an earlier study (6), the presence of Ad-36 DNA in the brains of the infected group warrants such an investigation in marmosets.

Considering the very small starting body weights of the marmosets, the mean post-inoculation weight gain of 41g represents a 12% increase in the Ad-36 group, which is approximately threefold greater than the 3.2% mean weight gain in the control group. Also, the Ad-36 group had an 11.5% total fat gain vs. 6.8% in the control group and 66% more visceral fat than the controls. Given the very small size of marmosets, these findings are of major biological importance.

The data presented in this paper suggest that Ad-36 plays a role in increasing body weight in nonhuman primates such as rhesus monkeys and marmosets; either animal could be used as a surrogate model with which to study the role of Ad-36 in human obesity. However, as a model, marmosets appear to have some advantages over rhesus monkeys because they are smaller, easier to house and handle and less expensive. In addition, in our initial screening of monkeys from the WRPRC colony for Ad-36 antibodies, < 4% of the marmosets were antibody positive. This very low prevalence of Ad-36 antibodies in marmosets gives a much better chance to select antibody-free marmosets for a prospective study (rhesus monkeys from the WRPRC had much greater and frequent contact with humans, whereas the marmosets are housed in a more controlled environment to prevent inadvertent infections). Additionally, the fact that marmosets have a life-expectancy of ~8 y (vs. 30–40 y for rhesus monkeys) means that in future prospective studies, marmosets could be tracked relatively easily during their complete life-cycle to ascertain the long-term consequences of Ad-36 infection.

A survey from three different states in the United States showed a 30% prevalence of Ad-36 antibodies in obese but only a 5% prevalence of the antibodies in nonobese subjects (13). The antibody-positive obese subjects also had significantly lower serum cholesterol levels (13). Body weight gain and hypocholesterolemia in response to Ad-36 infection in two divergent primate species support the hypothesis that the virus may play a causative role in human adiposity and demonstrate the suitability of the two animal models for further of the phenomenon. In addition, the relationship of Ad-36 with body composition and serum lipids in these models suggests that in the future, investigators studying body weight, obesity, obesity treatment or evaluation of serum lipids in rhesus monkeys or marmosets would be wise to take into account the status of infection with Ad-36 because Ad-36 may add extraneous variance to the outcome.

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Increased adiposity in animals due to a human virus

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BACKGROUND: Four animal models of virus-induced obesity including adiposity induced by an avian adenovirus have been described previously. This is the first report of adiposity induced in animals by a human virus.
OBJECTIVE: We investigated the adiposity promoting effect of a human adenovirus (Ad-36) in two different animal models.
DESIGN: Due to the novel nature of the findings we replicated the experiments using a chicken model three times and a mammal model once. In four separate experiments, chickens and mice were inoculated with human adenovirus Ad-36. Weight matched groups inoculated with tissue culture media were used as non-infected controls in each experiment. Ad-36 inoculated and uninfected control groups were housed in separate rooms under biosafety level 2 or better containment. The first experiment included an additional weight matched group of chickens that was inoculated with CELO (chick embryo lethal orphan virus), an avian adenovirus. Food intakes and body weights were measured weekly. At the time of sacrifice blood was drawn and visceral fat was carefully separated and weighed. Total body fat was determined by chemical extraction of carcass fat.
RESULTS: Animals inoculated with Ad-36 developed a syndrome of increased adipose tissue and paradoxically low levels of serum cholesterol and triglycerides. This syndrome was not seen in chickens inoculated with CELO virus. Sections of the brain and hypothalamus of Ad-36 inoculated animals did not show any overt histopathological changes. Ad-36 DNA could be detected in adipose tissue, but not skeletal muscles of randomly selected animals for as long as 16 weeks after Ad-36 inoculation.
CONCLUSIONS: Data from these animal models suggest that the role of viral disease in the etiology of human obesity must be considered.
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Keywords: serum cholesterol; serum triglycerides; body fat; body weight; obesity; visceral fat

Introduction

Obesity is a serious disease that affects more than one third of adults and over 20% of children in the USA and produces major increases in morbidity and mortality.[1,2] There are multiple etiologies of obesity, but most scientists in the field have focused either on genetic factors or behavioral aspects. Isolated cases of obesity due to hypothalamic damage from accidents, tumors, bacterial infections and other unusual events have been reported, but such cases have been thought to be exceedingly rare.[3]

No consideration has been given to the possibility that human obesity might be due to viral infections, despite the evidence that viral infections may cause obesity in animals. Four different animal viruses have been identified that produce obesity syndromes in animals. Lyons *et al*[4] reported that canine distemper virus produced obesity in mice and later studies suggested that the mechanism was hypothalamic damage due to the virus.[5–7] Rous associated virus type 7 (RAV-7) was reported to induce obesity in chickens.[8,9] RAV-7 virus-induced obesity was associated with stunting of growth, hyperlipidemia, fatty liver, hepatomegaly, anemia and immuno-suppression.[8,9] Borna disease virus, a single stranded RNA virus, produced a syndrome of obesity in rats characterized by lympho-monocytic inflammation of the hypothalamus, hyperplasia of pancreatic islets, and elevated serum glucose and triglyceride levels.[10]

Dhurandhar *et al*[11] reported that SMAM-1, a chicken adenovirus isolated in Bombay, India, produced excessive fat accumulation in the visceral depots and a paradoxical reduction of serum levels of cholesterol and triglycerides in chickens. Of 52 obese humans tested by agar gel-precipitation test, 10 had antibodies to SMAM-1.[12] These 10 individuals had a higher body weight and lower serum cholesterol and triglycerides compared to antibody negative individuals.[12]

We wished to further study the adiposity promoting effect of SMAM-1 in the USA. However, the United States Department of Agriculture refused permission to import SMAM-1 from India. This prompted us to

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investigate the adiposity-promoting potential of a human adenovirus that is available in the USA. There are currently 50 types of human adenoviruses deposited in the American Type Culture Collection (ATCC) virus bank. Human adenovirus-36 (Ad-36) was first isolated in 1978 in Germany from the feces of a girl with diabetes mellitus.[13] Ad-36 is serologically different from at least 47 of the other 49 human adenoviruses, as there is no cross-reaction of anti-bodies between Ad-36 and these other adenoviruses.[13-18] Ad-36 was our first candidate to investigate its adiposity-promoting potential mainly due to its antigenic uniqueness.

In the current study, we performed four experiments in animals: three in chickens, and one in mice, that demonstrate that Ad-36 increases adiposity in animals.

Methods

Four experiments involving two different animal species were performed to demonstrate the cross-species reproducibility of Ad-36 induced obesity: assays used are described under 'Assays and techniques' at the end of the Methods section.

Experiment 1—chickens

One-day-old specific pathogen free (SPF) white leghorn broiler chickens ($n=39$) were obtained from Specific Pathogen-Free Avian Supply (SPAFAS, Roanoke, IL) and were housed in the National Wildlife Health Center, Madison, WI, under Biosafety level 3 conditions, with a separate air supply to each room. Protective clothing, shoes, gloves, hairnets and masks were used to enter the rooms and utmost care was taken to prevent cross contamination.

Access to food (Purina Starter Grow) and water was provided *ad libitum* throughout the study period and food consumption, corrected for spillage, was recorded for individual cages. For the first 3 weeks, the chicks were housed in a brooder with a 12 h light cycle and a temperature of 95°F that was reduced gradually to 70°F at the end of 3 weeks. At 3 weeks of age, the chickens were removed from the brooder and maintained at 70 ± 2°F thereafter. Chickens were weighed at 1 week, 3 weeks, and then every week until the termination of the study at 6 weeks.

After 3 weeks, the chickens were divided into three weight matched groups of 13 each (Ad-36, CELO and control) and were housed in three separate rooms (two chickens/cage). Blood was drawn from a wing vein for baseline measurements of serum cholesterol and triglycerides, and for measurement of adenovirus antibodies to insure that the chickens had not been previously exposed to Ad-36. All these assays were repeated at the time of sacrifice.

Group 1, the control group, was inoculated intranasally (i.n.) with 0.2 ml of media. Group 2 was inoculated i.n. with 0.2 ml of a suspension of human adenovirus Ad-36, representing a dose of 10^5 PFUs (plaque forming units) of Ad-36 and the third group was inoculated i.n. with 0.2 ml of CELO virus (10^4 PFU). Throat and rectal swabs were taken from all chickens one week after virus inoculation to confirm infection by virus isolation.

Three weeks after inoculation the animals were fasted overnight and sacrificed. The omental-mesenteric (visceral) fat was carefully dissected from each bird and weighed. Six birds from each group were randomly selected for total carcass fat determination.

Experiment 2—chickens

The above experiment was repeated in 32 male SPF chickens. In this experiment chickens were observed for a longer period of time after the virus inoculation. Housing conditions were similar to those described in experiment 1. Two groups of weight-matched animals ($n=16$ per group) were used for this experiment. Animals were inoculated intranasally at 3 weeks of age (1.8×10^6 PFU for the Ad-36 group and 200 µl sterile media for the control group). Food intake was measured for each cage. Blood was drawn from a wing vein 36 h after inoculation to determine viremia by virus isolation from the blood sample. Blood was also drawn 10 days post inoculation and at the time of sacrifice. Chickens were sacrificed 5 weeks post inoculation, body weight measured, visceral fat was separated and weighed. About 1 g samples of visceral adipose tissue and skeletal muscle from breast muscle area (keel) were removed and flash frozen in liquid nitrogen for viral DNA detection using capillary electrophoresis assay. Serum cholesterol and triglycerides levels were determined in the final serum obtained.

Experiment 3—chickens

This experiment assessed a different route of infection (intraperitoneal *vs* intranasal inoculation), a longer time period before sacrifice, and histopathological examination of the brains. Housing conditions were similar to those described above. Three-week-old male SPF chickens ($n=10$) were inoculated intraperitoneally with 10^5 PFU of Ad-36 virus media (Ad-36 group) or 0.2 ml of sterile media ($n=8$). Chickens were sacrificed 13 weeks post inoculation, body weight was measured, and visceral fat was separated and weighed. One gram samples of visceral adipose tissue were removed and flash frozen in liquid nitrogen for viral DNA detection using capillary electrophoresis assay. Randomly selected samples (three from each group) were tested for the presence of Ad-36 DNA with the help of capillary electrophoresis assay. At sacrifice the brains were carefully removed, preserved in 30 times the volume of 37% formalin, and sectioned for histopathological examination. Serum cholesterol and triglycerides levels were determined in the final serum obtained.

Experiment 4—mice

This preliminary experiment was conducted to investigate the adipogenic effect of Ad-36 in a mammal model. Thirty-five 4-week-old outbred female ICR mice (Harlan Labs, Indianapolis) were received. Five mice were randomly chosen for sacrifice to draw blood for obtaining baseline serum. Baseline serum was obtained to ensure that the mice were free from Ad-36 antibodies at the beginning of the experiment. The remaining 30 mice were weight matched into two groups and injected i.p. with 0.2 ml of Ad-36 media containing 2×10^7 PFU ($n = 20$) or 0.2 ml of tissue culture media ($n = 10$, control group). Mice were housed under Biosafety level 3 containment, were offered *ad libitum* access to food and water, and food intakes and body weights were measured weekly. Blood samples for cholesterol, triglycerides and viral titers by serum neutralization were drawn at 10 weeks and at sacrifice 22 weeks post inoculation. At sacrifice, the visceral inguinal and retroperitoneal fat pads were dissected free and weighed. Body composition was performed on all animals.

Assays and techniques

Preparation of the virus suspension. Human adenovirus-36 (Ad-36, ATCC no. VR-913) and avian adenovirus CELO (ATCC no. VR 432) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The Ad-36 work stock was grown on A549 cells, a human bronchial carcinoma tissue culture line and the CELO work stock was grown using SL-29 cells (chick embryo fibroblasts). The titer of the work stocks that caused a cytopathic effect (CPE) in 50% of the wells containing A549 or SL-29 cells was calculated and expressed as tissue culture infectivity dose (TCID-50) units per ml. TCID-50 of the work stocks were determined using serial 10-fold dilutions of the virus work stock.

Plaque forming units assay. Titers of the plaque purified Ad-36 and CELO virus were determined in A549 and SL-29 cells, respectively, by this assay. The harvested and frozen virus suspensions were rapidly thawed at 37°C in a water bath. Starting with 100 µl of virus suspension and 900 µl of media, serial 10-fold dilutions were made. Cells were grown to confluence in six well plates and three wells were used for each dilution. Three wells were used as the blank control and were not infected with the virus suspension. The media was removed from each well and 100 µl of media containing serially diluted virus suspension were pipetted into the wells. The plates were incubated at 37°C, shaking gently every 15 min. After 1 h of incubation, viral suspension from the wells was removed and discarded. The wells were overlaid with about 3 ml of 1% agar in media per well, with 1 × antibiotic-antimycotic solution. The plates were inverted and incubated at 37°C for 8 days until plaques appeared. After 8 days, wells were stained overnight with about 1 ml of crystal violet per well. The next day, the number of plaques formed was counted after removing the agar. The number of plaques formed × dilution of viral suspension used gave PFU/100 µl of inoculum used. This was multiplied by 10 to express PFU/ml.

Serum neutralization assay for viral antibodies. Serum neutralization assays were performed using the 'constant virus-decreasing serum' method. Test serum was heat inactivated in a water bath at 57°C for 1 h and serially diluted (2-fold) in 96-well plates. The serum dilutions ranged from 1:2 to 1:512. One hundred TCID-50 of the respective adenovirus work stock was added to each of the wells. A549 cells (SL-29 cells were used for detecting CELO antibodies) were added to these wells after 1 h of incubation at 37°C. Each test serum was run in duplicate. Serum control (serum and cells, but no virus), cell control (cells alone, no virus, no serum) and virus control (cells and virus, no serum) were included with each assay. Plates were incubated at 37°C for 11 days and the presence of CPE was noted. Serum samples without CPE in dilutions of 1:8 or higher were considered positive for the presence of neutralizing antibodies to the respective virus. CPE in virus control wells, and its absence in serum control and cell control wells, was ascertained.

Virus isolation. A549 human bronchial carcinoma cells in tissue culture were used for isolation of Ad-36 and SL-29 cells were used for isolating CELO virus. Flasks containing A549 cells or SL-29 cells were inoculated with about 200 µl of sample (media from the oral-rectal swabs or whole blood) and were incubated at 37°C in media containing 2 × antibiotic–antimycotic solution. Culture media from the cells was collected after 8 days and transferred to cells in new set of flasks. This was repeated for a total of three passages. Infection was confirmed by observing if viral CPE occurred in the respective cells.

Assays for serum cholesterol and triglycerides. Serum cholesterol was assayed using the cholesterol oxidase-peroxidase method. Colorimetric determinations were made using Sigma kits (catalogue no. 352) and the absorbance read at 500 nm. Cholesterol calibrator (Sigma catalogue no. C 7921) and Cardiolipid control (Sigma catalogue no. C 4571) were used.

Triglycerides were determined using the glycerol phosphate–peroxidase method. Colorimetric determinations were made using Sigma kits (catalogue no. 339-50) and the absorbance was read at 540 nm. Glycerol (Sigma catalogue no. 339-11) was used as a standard.

Body composition analysis. Digestive tracts of the carcasses were cleaned and returned to the carcasses. After autoclaving and homogenization of the carcasses, aliquots were used for water, ash and fat contents. All measurements were performed in triplicate. Water content was determined by heating samples to a constant weight in a drying oven overnight at 90°C. Ash content was determined by incinerating the sample in a furnace at 600°C for 4 h. The Folch extraction method was used for body fat determination. Fat was extracted with methanol–chloroform.

Capillary electrophoresis. The assay has been previously described in detail.[19] Briefly, the assay was divided in three parts, namely Probe synthesis, DNA extraction and hybridization, and CE-LIF (capillary electrophoresis–laser-induced fluorescence) analysis.

Probe synthesis. We have the sequence of the entire Ad-36 genome. To ensure specificity of the DNA detection, only a segment with a sequence unique to Ad-36 was probed. The uniqueness of the sequence was verified by a gene bank search. A 5′fluorescein phosphoramidite labeled DNA probe for Ad-36 adenovirus was synthesized by IDT Laboratories (Coralville, IA).

DNA extraction and hybridization. The DNA from the sample was extracted with the Qiagen QIAmp Blood or tissue kit and quantitated spectrophotometrically. The genomic DNA was then digested with MboI by standard procedures to generate smaller DNA fragments for hybridization, and treated with RNAse One to remove any RNA contamination. This DNA was then hybridized with the DNA probe (1.0125 μg) in a buffer volume of 30 μl containing 10 mM Tris–HCl (pH 7.2), 1 mM EDTA (pH 8.0) and 50 nM NaCl. The mixture was heated at 85°C for 10 min, and then incubated at 42°C for 4 h. Following the incubation, samples were flash frozen at −80°C.

CE-LIF analysis. Separations were performed on a P/ACE 2050 system using laser-induced fluorescence in the reversed-polarity mode (anode at the detector side) at excitation of 488 nm and emission of 520 nm. Samples were introduced hydrodynamically by 10 s injections at 0.34 Pa across a 65 cm × 100 μm coated eCAP dsDNA capillary filled with replaced linear polyacrylamide. The capillary was conditioned with eCAP dsDNA 1000 gel buffer. Separations were performed under constant voltage at 7.0 kV for 15–30 min.

The hybridization procedure generates a DNA fragment comprising the fluorescently labeled probe and the target viral DNA. The negative control generates a DNA fragment of the probe bound to itself. The migration times of the positive and the negative control are nearly identical, however, the positive sample has an increased peak area indicating bound DNA and a slightly longer retention time due to a mass effect. Samples were quantitated by comparing the peak area obtained for the samples to a control from an identical tissue. The viral DNA is calculated based on the difference in peak areas. A linear relationship between the peak area and concentration was demonstrated from 0.072 to 21.46 pg ($r^2 = 0.99$, $y = 516.88 + 18.01x$). The intra-day and inter-day migration time precision was 0.18% ($n = 9$) and 0.22% ($n = 6$), respectively. The intra-day peak area precision was 7.3% ($n = 6$) and the inter-day peak area precision was 11% ($n = 9$). The minimal detectable level was 36 ag (signal-to-noise ratio 3:1).

DNA from A549 cells without virus, and DNA from A549 cells infected with Ad-36 were used as negative and positive controls, respectively.

Histopathology of brains

Specimens of brain were paraffin embedded, cut with a microtome, and stained with hematoxylin–eosin. One micron sections were taken to include the hypothalamus, and every fifth section was examined. About 15–30 sections were examined per chicken, of which at least two in each chicken were from the hypothalamus.

Statistical analysis

Means of groups were compared with those of the control using Student's *t*-test followed by Bonferroni adjustment for multiple comparisons. The difference in prevalence of obese animals in each group was tested by the chi-square test.

Results

Experiment 1

Confirmation of viral infection. Isolation of Ad-36 and CELO viruses was attempted using three passages on A549 or SL-29 cells. After three passages of oral–rectal swabs, Ad-36 and CELO viruses could be isolated from all animals of the respective groups. No virus could be isolated from the control group. Virus isolated from swabs from Ad-36 inoculated chickens demonstrated the presence of human adenovirus antigen by Adenoclone enzyme immunoassay (Meridian Diagnostics, Cincinnati), further confirming infection with this human virus. No EIA kit was available to detect CELO virus. Infection was also confirmed by a four-fold or more rise from baseline of specific neutralizing antibody titers in all animals from the Ad-36 and the CELO groups. No rises and no antibody titers were noted in the control group.

Body composition and prevalence of obesity. Post inoculation cumulative food intake per chicken was not different for the three groups (mean ± s.d., control group 1150.0 ± 157.0 g, Ad-36 group 1038.5 ± 249.1 g and CELO group 1282.5 ± 505.5 g; P = NS compared to the control group). Body weights were not different among the groups at the time of sacrifice, but visceral fat and total body fat were significantly greater ($P < 0.02$) in the Ad-36 chickens compared to controls (Table 1). Visceral fat of the Ad-36 group was 100% greater than that of the control group. Visceral fat and the carcass fat of the CELO group did not differ significantly from that of the control group (Table 1).

If obesity is defined as a visceral fat greater than the 85th percentile of the range of the control group, our data show that three chickens in the control group (23%) and nine in the Ad-36 group (70%) were obese ($P < 0.02$; Table 2). Six chickens from the CELO group (46%) were obese by the definition; this prevalence in the CELO group was not significantly different from that of the control group.

Serum lipids. Compared to the control, serum cholesterol was lower only for the Ad-36 group ($P < 0.02$), but serum triglycerides levels were significantly lower for the Ad-36 as well as the CELO group (Table 1).

Experiment 2

Confirmation of viral infection. Ad-36 virus could be isolated in tissue culture by repeated cell passages of the blood obtained 36 h post inoculation from the Ad-36 group and detected as a human adenovirus with the help of Adenoclone EIA kit in 12 out of 16 chickens. No virus could be isolated from the control group. Blood drawn 10 days post inoculation demonstrated neutralizing antibodies to Ad-36 in 10 of 16 chickens. None of the control chickens had such antibodies.

DNA isolated from the blood, visceral adipose tissue and skeletal muscle obtained from three infected and three control chickens were assayed for the presence of Ad-36 DNA by capillary electrophoresis.[19] Ad-36 DNA could be detected in all three blood samples drawn 36 h after infection and in two of three samples of visceral fat taken at sacrifice 5 weeks after infection, but not in the muscle samples taken at 5 weeks from the Ad-36 infected chickens. Ad-36 DNA was not detected in any tissues of the control chickens at any time.

Body composition and prevalence of obesity. Post inoculation cumulative food intake per chicken was not different for the two groups (mean ± s.d., control group 5401.6 ± 268.1 g, and Ad-36 group 5234.8 ± 338.3 g; P = NS). Body weights were not different among groups, but visceral fat was 128% higher in the Ad-36 chickens ($P < 0.0005$; Table 3). Total body fat was 46% greater in the Ad-36 infected group, compared to the control ($P < 0.0005$).

Using the definition of obesity as a total body fat greater than the 85th percentile of the range of the control group, 64% of Ad-36 chickens and 18% of control chickens were obese ($P < 0.02$; Table 2).

Serum lipids. Compared to the control group, serum cholesterol and triglycerides were lower ($P < 0.0005$ and $P < 0.02$, respectively) in the Ad-36 group (Table 3).

Experiment 3

Confirmation of viral infection. Serum neutralization assays of the kill serum confirmed the presence of antibodies to Ad-36 in all chickens in the Ad-36 group and none in the control group. Capillary electrophoresis demonstrated Ad-36 DNA in visceral fat of all three infected chickens tested, but in none of the controls.

Table 1 Experiment 1: infection of chickens with human adenovirus Ad-36

	Control	*Ad-36*	*CELO*
Number	13	13	13
Body weight (g)	502 ± 18.2	538 ± 18.2	527 ± 16
Visceral fat (%)	0.27 ± 0.05	0.54 ± 0.07*	0.37 ± 0.04
Total body fat (%)	5.8 ± 0.6	6.9 ± 0.1*	5.9 ± 0.6
Cholesterol (mmol/l)	7.14 ± 0.57	4.53 ± 0.31*	5.58 ± 0.61
Triglycerides (mmol/l)	0.84 ± 0.05	0.69 ± 0.03**	0.68 ± 0.02*

Mean ± s.e.m.; *$P \leq 0.02$; **$P < 0.05$ compared to control.

Table 2 Prevalence of obesity (%)

	Control	*Ad-36*	*CELO*
Experiment 1 (chickens)	23.07	69.23*	46.15
Experiment 2 (chickens)	18.1	63.6*	
Experiment 3 (chickens)	12.5	70.0*	
Experiment 4 (mice)	22.22	60.0**	

*$P < 0.02$; **$P < 0.05$. Obesity was defined as greater than the 85th percentile of adiposity of the control group.

Table 3 Experiment 2: replication of Ad-36 induced obesity in chickens

	Control	*Ad-36*
Number	16	16
Body weight (g)	1250 ± 28.4	1328 ± 39.4
Visceral fat (%)	1.08 ± 0.14	2.47 ± 0.23*
Total body fat (%)	7.8 ± 0.5	11.4 ± 0.6*
Cholesterol (mmol/l)	3.54 ± 0.10	3.03 ± 0.10*
Triglycerides (mmol/l)	1.13 ± 0.09	0.88 ± 0.04**

Mean ± s.e.m.; *$P \leq 0.0005$; **$P < 0.02$ compared to control.

Body composition and prevalence of obesity. Body weights and post inoculation cumulative food intakes were not different between Ad-36 and control groups, but visceral fat was 74% greater in the Ad-36 group (1.74±0.2% *vs* 1.01±0.2%, $P=0.03$; Table 4).

Seventy percent of the infected chickens (seven out of 10) had visceral fat weights above the 85th percentile of the range of the control group, whereas 12.5% of control chickens (one out of eight) met this criteria ($P<0.02$; Table 2).

Serum lipids. Compared to the control group, serum cholesterol and triglycerides of the Ad-36 group were lower by 20% and 18%, respectively. However, the values did not reach statistical significance.

Brain histopathology. The sections revealed no morphological abnormalities in either group, with the exception that one sample from each group had changes thought to be not relevant (as described below). One control chicken had a unilateral focus of mild gliosis and perivascular accumulation of lymphocytes in the pituitary isthmus of the hypothalamus and some vacuolization of the myelin sheath in some areas of white matter. In one Ad-36 infected chicken, a single dilated blood vessel with transmural localization of mononucleated cells was noted adjacent to the third ventricle.

Experiment 4

Confirmation of viral infection. Absence at baseline of antibodies to Ad-36 was confirmed in five randomly selected mice by serum neutralization assay. Ad-36 antibodies were seen in 12 of 20 Ad-36 inoculated mice after 10 weeks and in 19 of 20 mice after 22 weeks. None of the control mice had a positive Ad-36 antibody titer (≥1:8).

Body composition and prevalence of obesity. One animal from the control group, who was negative for antibodies to Ad-36, had a very high body weight (38.8 g) and visceral fat (2.43 g). This animal qualified as an outlier by two separate statistical tests for data rejection (Q Distribution Deviate and Maximum Normal Residual test). The data from this animal were discarded. Post inoculation cumulative food intake per animal was not different for the two groups (mean±s.d., control group 467.0±22.7 g, and Ad-36 group 480.3±33.7 g; P=NS). Compared to the control group, the mean body weight was 9% greater in Ad-36 mice ($P<0.05$), total body fat was 35% greater ($P<0.02$), and visceral fat was 67% greater (1.0 g *vs* 0.6 g; $P<0.02$; Table 5). Retroperitoneal and inguinal fat pads were not different for the two groups.

Sixty percent of mice (12 out of 20) infected with Ad-36 and 22% of the control mice (two out of nine; $P<0.02$) had total percent body fat weights above the 85th percentile of the range of the control group (Table 2).

Serum lipids. Serum cholesterol and triglycerides in the Ad-36 group were significantly lower than control by 38% ($P<0.03$) and 31% ($P<0.008$), respectively (Table 5).

Discussion

Four experiments demonstrated that Ad-36 increased visceral fat, total fat, and/or body weight compared to the control group. The first experiment tested the adiposity promoting effect of CELO virus along with Ad-36. CELO is antigenically similar to SMAM-1 and present in the USA and, therefore, we chose to use CELO in Experiment 1. Asymptomatic presence of CELO virus was reported from 64% to 100% of apparently healthy chickens 5 weeks of age and older.[20] The first experiment demonstrated that CELO, an avian adenovirus, did not produce adiposity similar to that produced by Ad-36 and that the adipogenic effect of Ad-36 may not be common to all adenoviruses.

Experiment 2 confirmed the findings that Ad-36 inoculation leads to increased visceral and total adiposity and paradoxically lower serum cholesterol and triglycerides. Experiment 3 was undertaken primarily to assess the effect of Ad-36 inoculation on the hypothalamus, as hypothalamic damage has been

Table 4 Experiment 3: intraperitoneal inoculation of Ad-36 in chickens

	Control	*Ad-36*
Number	8	10
Body weight (g)	1533.5±59.1	1625.4±53.8
Visceral fat (%)	1.01±0.19	1.74±0.25*
Cholesterol (mmol/l)	3.04±0.36	2.51±0.26
Triglycerides (mmol/l)	2.35±0.24	1.92±0.13

Mean±s.e.m.; *$P\leq0.04$ compared to control.

Table 5 Experiment 4: infection of mice with Ad-36

	Control	*Ad-36*
Number	9	20
Body weight (g)	27.9±0.79	30.5±0.95*
Visceral fat (%)	2.13±0.27	3.16±0.27**
Visceral fat (g)	0.60±0.08	1.00±0.11**
Inguinal fat (g)	0.22±0.02	0.25±0.02
Retroperitoneal fat (g)	0.30±0.03	0.27±0.03
Total fat (%)	7.2±0.57	9.3±0.6**
Cholesterol (mmol/l)	2.68±0.35	1.67±0.08**
Triglycerides (mmol/l)	2.26±0.18	1.56±0.06*

Mean±s.e.m.; *$P\leq0.05$; **$P<0.03$; *$P<0.008$ compared to control.

postulated to be the cause of obesity induced by canine distemper virus and borna disease virus[4–7,10] Experiment 4 was a preliminary experiment to test the suitability of a mammal model for future studies involving adiposity promoting effect of Ad-36.

Previously, human and avian adenoviruses were not thought to infect across species. The isolation of Ad-36 from oral and rectal swabs taken from infected animals 7–10 days after infection, as well from the blood drawn 36 h post inoculation of the infected animal and the presence of viral DNA in adipose tissue as long as 16 weeks post-inoculation are unequivocal evidence of cross-species infection with this human virus. Four-fold or greater change in the antibody titer is considered an evidence of infection. Twelve mice from the Ad-36 group showed ≥4-fold rise in antibody titer between 10 and 22 weeks post inoculation. Although this observation suggests virus replication in animals, we cannot explain the observation at this time.

Ad-36 did not cause severe symptoms in any of the experiments. Some animals appeared to have reduced activity for 1–2 days post-inoculation, but no other overt signs or symptoms were noted and there was no premature mortality in any of the experiments.

Both intra-nasal and intra-peritoneal inoculation of Ad-36 increased adiposity, predominantly by increasing visceral adipose tissue stores. Individual susceptibility to increased adiposity was different among animals. Using the 85th percentile for adiposity of the control group as an arbitrary definition of obesity, 60–70% of all Ad-36 inoculated animals in each experiment became obese.

Different times of sacrifice post inoculation revealed that increased adiposity could be observed as soon as 3 weeks (experiment 1), was not of transient nature, and persisted for 13 weeks (experiment 3) to 22 weeks (mice—experiment 4). Durations selected in this study should be helpful for future work in the area.

The mechanisms of obesity with Ad-36 are unknown. Histopathological examination of the brains of chickens did not show any evidence of damage to the hypothalamus or other areas of the brain. Hypothalamic damage was postulated to be the etiology of the obesity after infection with canine distemper virus and borna disease virus[5–7,10] However, the initial report of canine distemper did not observe any hypothalamic damage,[4] and subsequent reports demonstrated that the timing of the sacrifice and examination of the brains was critical in demonstrating the presence of virus in the hypothalamus.[5–7] We used only one time point, 13 weeks after infection in chickens. It is possible that we missed the critical period, and more studies are needed with sacrifice at intervals after infection.

In addition to lesions of the brain, abnormalities of peripheral mechanisms may be postulated to produce obesity. Ad-36 DNA was detected by capillary electrophoresis in adipose tissue at 5 weeks and at 13 weeks after initial infection in chickens. The paradoxical reduction of serum cholesterol and triglycerides seems unlikely to be due to a central mechanism, but would support the postulate of an abnormality in adipose tissue. The presence of adiposity and the presence of the lower serum lipids appear to be linked and, if so, future research probably should focus on peripheral mechanisms. The liver would be the next obvious organ to investigate for a possible role in the observed syndrome. Conversely, it is also possible that the two conditions may not be linked, but may operate through separate mechanisms. It is unlikely that the reductions are due to non-specific mechanisms such as increased cytokines. For example, gamma-interferon can reduce serum cholesterol,[21] but such reductions are transient and are usually accompanied by increased, rather than decreased, serum triglycerides levels. Recently, we observed increased adipocyte differentiation due to Ad-36 *in vivo* as well as *in vitro*.[22] Three times as many 3T3L-1 preadipocytes differentiated when treated with Ad-36, compared to the uninfected control 3T3l-1 cells. Another human adenovirus, Ad-2, did not show increased differentiation of 3T3L-1 preadipocytes under similar conditions.[22] The effect of Ad-36 on adipocyte differentiation suggests that Ad-36 induces adipocity by targeting adipocytes. Effect of Ad-36 on adipocyte differentiation needs to be further characterized.

This study is the first report to demonstrate that a human virus can produce increased adipose tissue in animals. It is possible that other human adenoviruses may increase adiposity in animals, but the failure of CELO virus to significantly increase adiposity suggests that this property differs among adenoviruses. Regardless of the fact that the mechanism of action as well as the adipogenic potential of other human viruses is unknown, the finding that a human virus induces adiposity in animals is an unusual and potentially significant finding.

There is little information about the epidemiology, pathophysiology and the usual route of infection of Ad-36. Human adenoviruses are associated with infection of the respiratory tract, gastrointestinal tract, and conjunctiva. The initial isolation of Ad-36 from feces of a diabetic child with enteritis suggests that this virus may be spread by the oral–fecal route, but there is insufficient evidence to draw conclusions about other routes of entry.

The finding of low serum cholesterol and triglycerides provides a convenient marker for this syndrome. Patients previously reported to have antibodies to the avian adenovirus, SMAM-1, also had low serum lipids.[12] About 30% of the obese individuals but only 5% of non-obese individuals have antibodies to Ad-36 and the obese antibody positive individuals have significantly lower serum cholesterol and triglycerides.[23] These observations from different experiments, when taken together, suggest that there may be some fundamental property of some adenoviruses that

predisposes infected animals, and perhaps infected people, to obesity and altered serum lipids. Additional research using other adenoviruses is needed.

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PAPER

Transmissibility of adenovirus-induced adiposity in a chicken model

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BACKGROUND: We previously reported that human adenovirus Ad-36 induces adiposity and paradoxically lower levels of serum cholesterol (CHOL) and triglycerides (TG) in animals.
OBJECTIVE: To evaluate the transmissibility of Ad-36 and Ad-36 induced adiposity using a chicken model.
DESIGN: *Experiment 1*—four chickens were housed (two per cage) and one from each cage was inoculated with Ad-36. Duration of presence of Ad-36 DNA in the blood of all chickens was monitored. *Experiment 2*—two groups of chickens were intranasally inoculated with Ad-36 (infected donors, I-D) or media (control donors, C-D). Blood drawn 36 h later from I-D and C-D groups was inoculated into wing veins of recipient chickens (infected receivers, I-R, and control receivers, C-R, respectively). On sacrifice, 5 weeks post-inoculation, blood was drawn, body weight noted and visceral fat was separated and weighed.
RESULTS: *Experiment 1*—Ad-36 DNA appeared in the blood of the inoculated chickens and that of uninoculated chickens (cage mates) within 12 h of inoculation and the viral DNA persisted up to 25 days in the blood. *Experiment 2*—compared with C-D, visceral and total body fat were significantly greater and CHOL significantly lower for the I-D and I-R. TG were significantly lower for the I-D. Ad-36 was isolated from 12 out of 16 blood samples of the I-D that were used for inoculating I-R chickens. Ad-36 DNA was present in the blood and the adipose tissue of the I-D and I-R but not in the skeletal muscles of animals selected randomly for testing.
CONCLUSION: As seen in experiment 1, Ad-36 infection can be transmitted horizontally from an infected chicken to another chicken sharing the cage. Additionally, experiment 2 demonstrated blood-borne transmission of Ad-36-induced adiposity in chickens. Transmissibility of Ad-36-induced adiposity in chicken model raises serious concerns about such a possibility in humans that needs further investigation.
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Keywords: cholesterol; triglycerides; body fat; visceral fat; Ad-36; obesity

Introduction

Obesity has been called the number one public health problem in America.[1] Although obesity is recognized as a disease of multiple etiologies, a virus infection as an etiological factor has been ignored until now. Five different viruses have been shown to cause obesity in animal models,[2–8] but until recently there was no evidence to suspect that viruses might play a role in the etiology of human obesity. We have shown that Ad-36, a human adenovirus, produces adiposity and paradoxically low levels of serum cholesterol (CHOL) and triglycerides (TG) in animal models,[8] and that a unique syndrome consisting of low serum CHOL and TG levels is present in about 30% of obese humans subjects from three different states (Wisconsin, Florida and New York) who have antibodies to Ad-36.[9,10] Only 5% of the lean humans tested to date from the three states had antibodies to Ad-36, suggesting that infection with this virus carries a high probability of association with obesity.[10] The possible link between a virus and obesity in humans warrants serious investigation of the adiposity-promoting effect of this virus. For ethical reasons, we cannot infect humans to

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study the virus-induced adiposity and, therefore, we have used animal models to understand the characteristics of Ad-36 induced adiposity. Chickens develop Ad-36-induced adiposity in as little as 3 weeks[8] and therefore we have used a chicken model in this experiment.

Not much is known about the transmissibility of Ad-36 and Ad-36 induced adiposity. Blood is one of the possible routes of viral transmission. This paper describes two experiments which address the issue of Ad-36 transmission in a chicken model.

Methods

Experiment 1

The purpose of this experiment was to investigate whether Ad-36 inoculation is followed by appearance of the viral titers in the blood and to determine if exposure by close contact with Ad-36-infected animals produces infection. Four specific pathogen free (SPF) male chickens (1 day old) were obtained from Specific Pathogen-Free Avian Supply (SPAFAS, Roanoke, IL). At 3 weeks of age they were housed two per cage and one chicken from each cage was inoculated intranasally (i.n.) with Ad-36 (4×10^6 plaque forming units, PFU). The other chicken from each cage was not inoculated and served as a chicken 'in-contact' with the Ad-36 inoculated chicken. Blood was drawn from all the chickens before inoculation and 12 h, 36 h, 4 days, 11 days, 18 days and 25 days post inoculation. DNA obtained from the blood was screened for the presence of Ad-36 DNA using capillary electrophoresis technique described below.

Experiment 2

This experiment tested the possibility of blood-borne transmission of infection and of Ad-36-associated adiposity. One-day-old male SPF white leghorn broiler chickens ($n=64$) were obtained from SPAFAS and were housed under Biosafety level 2 conditions, with negative air pressure in each room. Food (Purina Starter Grow) and water were provided *ad libitum* throughout the study period and food consumption, corrected for spillage, was recorded for individual cages. For the first 3 weeks, the chicks were housed in a brooder with a 12 h light cycle and a temperature of 95°F that was reduced gradually to 70°F at the end of 3 weeks. At 3 weeks of age, the chickens were removed from the brooder and maintained at 70±2°F thereafter. Chickens were weighed at 1 week, 3 weeks, and then every week until the termination of the study at 8 weeks.

After 3 weeks, the chickens were divided into four weight-matched groups of 16 each. Blood was drawn from a wing vein for measurement of adenovirus antibodies to insure that the chickens had not been previously exposed to Ad-36. These assays were repeated at the time of sacrifice. At 4 weeks of age one group (infected donors, I-D) was inoculated i.n. with 200 µl of Ad-36 virus suspension (1.8×10^6 PFU). A second group (control donors, C-D) was inoculated i.n. with 200 µl sterile media. After 36 h, blood was drawn from the wing veins of the chickens from I-D and C-D groups and 200 µl blood was injected into the wing veins of I-R (infected receivers) and C-R (control receivers) groups, respectively. About 400 µl blood from I-D and I-R groups was also used to isolate the virus. Blood was drawn 10 days post inoculation to screen for Ad-36 antibodies.

Five weeks after inoculation the animals were fasted overnight and sacrificed. The omental–mesenteric (visceral) fat from each bird was carefully dissected and weighed. Body weights were measured and blood was drawn to determine CHOL and TG. About 1 g each of visceral fat and skeletal muscle from the right side of the keel bone (breast muscle) were flash frozen in liquid nitrogen. Total fat content of the carcasses of 11 chickens from each group was determined using the Folch extraction method.[11] DNA obtained from blood, visceral adipose tissue and skeletal muscle from three chickens each from the four groups was assayed for Ad-36 DNA by capillary electrophoresis assay.

Statistical analysis

In experiment 1, log (number of Ad-36 DNA copies per ml of blood) was plotted against the time points. In experiment 2, student's *t*-test followed by Bonferroni adjustments were used to analyze the differences in the means. Chi square test was used to analyze the difference in the prevalence of obesity in the four groups.

Techniques and assays

Utilization of the virus

Media used for tissue culture. Minimum Essential Media Eagle (MEM) (catalog no. M-0643, Sigma Chemicals) with non-essential amino acids, Earle's salts and *L*-glutamine was used for growing A549 cells for tissue culture. MEM with 10% fetal bovine serum (FBS), 2.9% $NaHCO_3$ (v/v) pH 7.4, was used for growing A549 cells, and for harvesting or isolating the virus.

Preparation of plaque-purified Ad-36 (Ad-36P). Human adenovirus-36 (Ad-36, ATCC no. VR-913) was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and the work stock was grown on A549 cells, a human bronchial carcinoma tissue culture line. This virus was plaque purified as described below to obtain genetic homogeneity.

Two hundred microliters of the 10^{-4} dilution of the virus work stock was inoculated in two wells of a six-well plate. Two wells of the plate were inoculated with 200 µl of media as negative controls. Plates were overlaid with 2 ml of 1% agar in media in each well, and the plates were inverted and incubated at 37°C till the plaques appeared. Under a microscope cells from a single plaque were aspirated using a micropipette, placed in 200 µl of media, and diluted 100-fold. This was considered the first passage. The diluted virus

solution was once again grown on A549 cells in a six-well plate and the procedure to select a single plaque was repeated. A total of three such passages were done and the resultant purified virus work stock was grown and was used for all the experiments. This purified Ad-36 tested positive with an enzyme immuno-assay (Adenoclone EIA, Meridian Diagnostics, Cincinnati, OH) that detects human adenoviruses. A PFU assay was done to determine the viral dose in the harvested material.

Preparation of the viral suspension. The plaque-purified virus was grown on A549 cells in 75 cm^2 tissue culture flasks at 0.1 multiplicity of infection and 90% destruction of the cells was observed due to cytopathic effect (CPE) after about 10 days. At this time, the flasks were frozen at −80°C and later thawed to lyse the cells and release the virus. The cell suspension was then centrifuged to pellet the cell debris and the clear supernatant was used as the viral suspension for inoculating the animals.

Plaque forming units assay. Titers of Ad-36 virus were determined using A549 cells by this assay. Starting with 100 µl of virus suspension and 900 µl of media, serial 10-fold dilutions were made. A549 cells were grown to confluence in six-well plates and three wells were used for each dilution. Three wells were used as the blank control and were not infected with the virus suspension. Media was removed from each well and 100 µl of the serially diluted virus suspension were pipetted out in the wells. The plates were incubated at 37°C, shaking gently every 15 min. After 1 h of incubation, the viral suspension from the wells was removed and discarded. The wells were overlaid with about 3 ml of 1% agar in media per well, with 1×antibiotic–antimycotic solution. The plates were inverted and incubated at 37°C for 8 days until plaques appear. After 8 days, wells were stained overnight with about 1 ml of crystal violet per well. The next day, number of plaques formed was counted after removing the agar. The number of plaques formed×dilution of viral suspension used gave PFU/100 µl of inoculum used. This was multiplied by 10 to express PFU/ml.

Tissue culture infectivity dose. The titer of the work stocks that caused a CPE in 50% of the wells containing A549 cells were calculated and expressed as tissue culture infectivity dose (TCID-50) units per ml. TCID-50 of the work stocks were determined using serial 10-fold dilutions of the virus work stock. TCID50 was calculated by serially diluting the virus stock solution and inoculating cells with the dilutions to find out the reciprocal of the highest dilution of virus which causes CPE in 50% of the cells inoculated. Titers were calculated after the cells inoculated with the virus dilutions were incubated at 37°C for 8 days.

Virus isolation

A549 cells in tissue culture were used for isolation of Ad-36 from the blood samples drawn 36 h post-inoculation of I-D and C-D groups. Flasks containing A549 cells were inoculated with about 200 µl of whole blood samples and were incubated at 37°C in MEM media containing 2×antibiotic–antimycotic solution and 10% FBS. Culture media from the flasks was collected after 8 days and transferred to cells in new set of flasks. This was repeated for a total of three passages. Infection was confirmed by observing whether viral CPE occurred in the respective cells 8 days after inoculation of the flask. The isolated virus was confirmed to be a human adenovirus by using a human adenovirus specific enzyme immuno-assay kit (Adenoclone EIA, Meridian Diagnostics, Cincinnati, OH).

Capillary electrophoresis assay

This assay is used to directly detect Ad-36 DNA in the blood.[12,13] Briefly, the assay is divided into three parts namely, probe synthesis, DNA extraction and hybridization, and CE-LIF (capillary electrophoresis-laser-induced fluorescence) analysis.

Probe synthesis. We have the entire Ad-36 genome sequenced. To ensure specificity of the DNA detection only a sequence unique to Ad-36 from the fiber protein region of the viral genome was probed. Uniqueness of the sequence was verified by a gene bank search. A 5 fluorscein phosphoramidite labeled DNA probe for Ad-36 adenovirus (5′ AGT TGA AAC AGC AAG AGA CTC AAA G) was synthesized by IDT Laboratories (Coralville, IA).

DNA extraction and hybridization. The DNA from the blood, adipose tissue and muscle samples of chickens was extracted with the Qiagen QIAmp Blood or tissue kit and quantitated spectrophotometrically. The genomic DNA was then digested with *MboI* (restriction enzyme from bacteria Moraxella Bovis) by standard procedures to generate smaller DNA fragments for hybridization, and treated with RNAse One to remove any RNA contamination. This DNA was then hybridized with the DNA probe (1.0125 µg) in a buffer volume of 30 µl containing 10 mM Tris–HCl (pH 7.2), 1 mM EDTA (pH 8.0) and 50 nM NaCl. The mixture was heated at 85°C for 10 min, and then incubated at 42°C for 4 h. Following the incubation, samples were flash frozen at −80°C.

CE-LIF analysis

Separations were performed on a P/ACE 2050 system using LIF in the reversed-polarity mode (anode at the detector side) at excitation of 488 nm and emission of 520 nm. Samples were introduced hydrodynamically by 10 s injections at 0.34 Pa pressure across a 65 cm×100 µM coated eCAP double stranded DNA (dsDNA) capillary filled with replaced

linear polyacrylamide. The capillary was conditioned with eCAP dsDNA 1000 gel buffer. Separations were performed under constant voltage at 7.0 kV for 15–30 min. The intra-day and inter-day migration time precision was 0.18% ($n=9$) and 0.22% ($n=6$), respectively. The intra-day peak area precision was 7.3% ($n=6$) and the inter-day peak area precision was 11% ($n=9$). The minimal detectable level was 36 ag (signal to noise ratio 3:1).

Data are obtained as relative fluorescence units (RFU), which are converted to picogram DNA by comparing the RFU obtained from a standard (pGEM Molecular Markers, catalog no. G174, Promega, Madison, WI) of known concentration picogram DNA. Viral loads in the blood samples were determined by comparison to a standard curve as previously described[12] and by expressing picogram DNA as number of copies of the virus. Briefly, one copy of Ad-36 DNA contains 33 068 base pairs. One picogram of DNA contains 9×10^8 bp, making 1 pg equal to approximately 27 216 copies of Ad-36 DNA. Results of the capillary electrophoresis assay (picogram of viral DNA) were expressed as the number of copies of Ad-36 per ml of the blood samples obtained from the two inoculated and two in-contact chickens before and after the viral inoculation (Figure 1).

DNA from A549 cells without virus, and DNA from A549 cells infected with Ad-36 were used as negative and positive controls, respectively. Also, DNA from A549 cells infected with other human adenoviruses Ad-2, Ad-31 and Ad-37 were used as negative controls.

Serum neutralization assay

This assay was used to screen the serum for Ad-36 antibodies. Serum was heat inactivated for 30 min at 56°C. The assay was carried out using 96-well microtiter plates. Serial 2-fold dilutions (1:2 to 1:1024) of serum were made with media and 50 µl of each dilution were added per well in duplicate. Fifty microliters of Ad-36 virus (100 TCID50) were added to each dilution. The plates were incubated at 37°C for 1 h. One hundred microliters of cell suspension containing about 20 000 cells were added to each well and the plates were incubated at 37°C for 12 days. Crystal violet-ethanol was added to each well to fix and stain the cells and the plates examined for CPE. The highest serum dilution with no CPE was considered the titer. Controls included wells containing no virus and/or wells with virus but no serum. A back titration to confirm the use of the appropriate virus dilutions

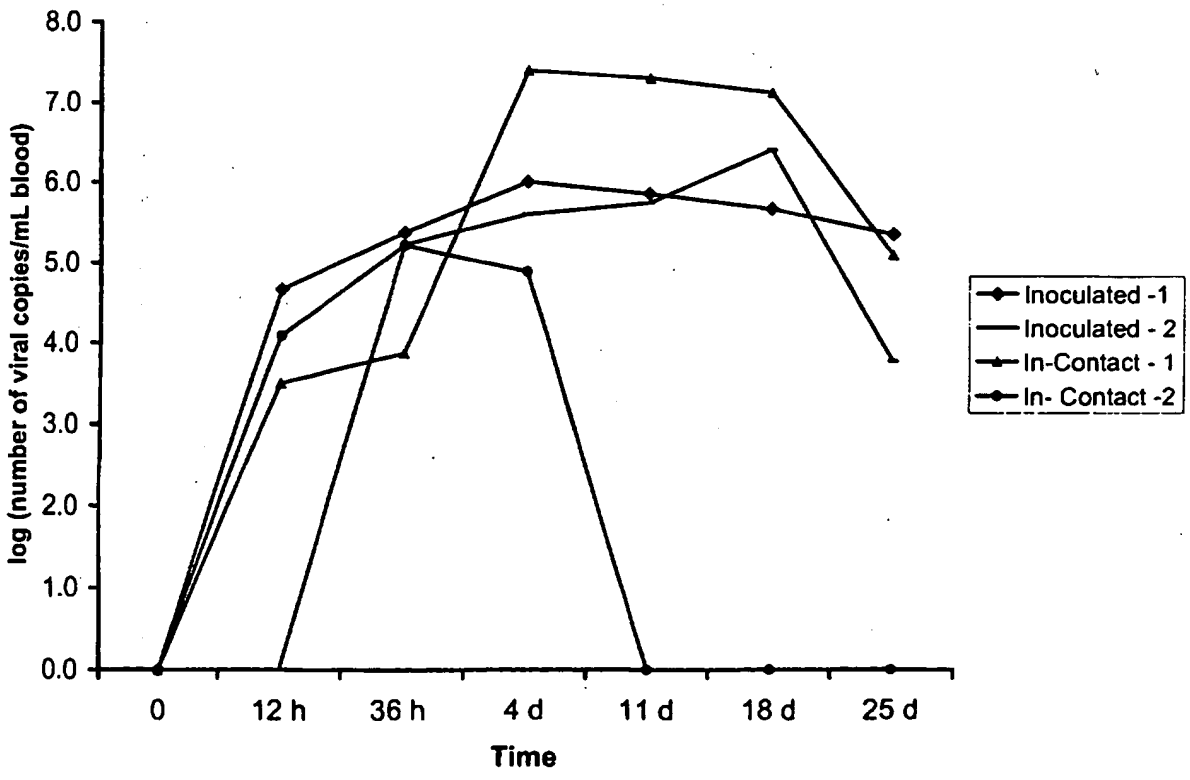

Figure 1 Ad-36 DNA titers in the blood of chickens inoculated with Ad-36 as well as the chickens kept in contact with the inoculated chickens. Time 0 denotes blood sample obtained before the viral inoculation.

was included. Presence of CPE with the virus and none in the presence of serum was considered an indication of effective neutralization of the virus with antibody in serum and the serum was considered to have antibody against the virus. A titer lower than 1:8 was considered to be non-specific neutralization and a titer of $\geq$ 1:8 was considered positive.

Assays for serum cholesterol and triglycerides

Serum CHOL was assayed using the CHOL oxidase-peroxidase method. Colorimetric determinations were made using Sigma kits (catalog no. 352) and the absorbance read at 500 nm. Cholesterol calibrator (Sigma catalog no. C 7921) and Cardiolipid control (Sigma catalog no. C 4571) were used.

TG were determined using the glycerol phosphate-peroxidase method. Colorimetric determinations were made using Sigma kits (catalog no. 339-50) and the absorbance was read at 540 nm. Glycerol (Sigma catalog no. 339-11) was used as a standard.

Body composition analysis

Digestive tracts of the carcasses were cleaned and returned to the carcasses. After autoclaving and homogenization of the carcasses, aliquots were used for water, ash and fat contents. All measurements were performed in triplicate. Water content was determined by heating samples to a constant weight in a drying oven overnight at 90°C. Ash content was determined by incinerating the sample in a furnace at 600°C for 4 h. The Folch extraction method[11] was used for body fat determination. Fat was extracted with methanol–chloroform.

Results

Experiment 1

Onset and duration of presence of Ad-36 DNA in the blood. Ad-36 DNA was absent in the blood of all four chickens drawn prior to the viral inoculation (0 time, Figure 1). Ad-36 DNA appeared in the blood 12–36 h post-inoculation in the chickens receiving Ad-36 inoculation and was detectable up to 25 days when the last blood sample was obtained (Figure 1). Ad-36 DNA was detectable in 12 h in both the 'in-contact' chickens. One of the 'in-contact' chickens had Ad-36 DNA up to 25 days and the DNA was undetectable at 11 days in the other 'in-contact' chicken.

Experiment 2

Transmission of Ad-36-induced adiposity by transfusion of blood obtained from Ad-36 infected animals. Detection of Ad-36. The virus could be isolated from 12 out of 16 blood samples of the I-D group that were used for inoculating I-R chickens, confirming active viremia at transfusion. The Adenoclone EIA kit confirmed that the isolated virus was a human adenovirus. No virus was isolated from the blood from the C-D group. Three animals from each of the four groups were randomly selected to test for Ad-36 DNA in the blood, adipose tissue and the skeletal muscle. Ad-36 DNA was detected in the DNA obtained from the blood and the adipose tissue of the I-D and I-R groups but not in the skeletal muscle DNA from these groups (Table 1). Ad-36 DNA could not be detected in the blood, adipose tissue or skeletal muscle of the control groups (C-D and C-R).

Antibodies to Ad-36. Ad-36 antibodies were absent in all chickens at the start of the experiment. Ad-36 neutralizing antibodies were detected in all chickens from the infected groups (I-D and I-R) 10 days after the viral inoculation and at sacrifice. Ad-36 antibodies were not detected in the control groups (C-D and C-R).

Body composition. Total cumulative food intake per chicken after the first inoculation was not different for any of the groups (mean ± s.d.; C-D, 5401.6 ± 268.1 g; C-R, 5585.5 ± 560.0 g; I-D, 5234.6 ± 338.3 g; I-R, 5316.2 ± 687.7 g, $P = 0.55$ by ANOVA). Mean body weights, visceral fat, total body fat (%), CHOL and TG levels or the prevalence of obesity were not different for the two control groups (C-D and C-R, Table 2). Compared to the C-D, final mean body weights of the I-D and I-R groups were not significantly different. Compared to the C-D group, visceral fat was greater by 142% and 80% for I-D ($P < 0.002$) and I-R ($P < 0.01$) groups, respectively (Table 2).

Compared to the C-D group, total carcass fat was greater by 35% for the I-D group ($P < 0.01$) and by 44% for the I-R

Table 1 Experiment 2: capillary electrophoretic detection of Ad-36 DNA in chicken blood and tissue obtained 5 weeks post-inoculation from control donors (C-D), control receivers (C-R), infected donors (I-D) and infected receivers (I-R)

	C-D	C-R	I-D	I-R
Number	3	3	3	3
Blood	−	−	+	+
Adipose tissue	−	−	+	+
Skeletal muscle	−	−	−	−

+, Ad-36 DNA present; −, Ad-36 DNA not detected.

Table 2 Effect of blood transfusion from Ad-36 inoculated chickens (I-D) and chicken inoculated with media (C-D) to infected receivers (I-R) and control receivers (C-R), respectively (Experiment 2)

	C-D	C-R	I-D	I-R
Number	16	16	16	16
Body weight (g)	1250 ± 28.4	1264 ± 15.6	1328 ± 39.4	1343 ± 28.6
Visceral fat (g)	13.7 ± 7.5	18.1 ± 8.2	33.1 ± 13.8†	24.7 ± 11.5‡
Body fat (%)	7.8 ± 0.5	8.8 ± 0.7	10.5 ± 0.7‡	11.2 ± 0.8‡
CHOL (mmol/l)	3.54 ± 0.1	3.56 ± 0.12	3.02 ± 0.09†	3.10 ± 0.13*
TG (mmol/l)	1.12 ± 0.08	1.01 ± 0.04	0.88 ± 0.04*	1.01 ± 0.05

Means ± s.e.m.
*$P \leq 0.045$; †$P \leq 0.002$; ‡$P \leq 0.01$.

group ($P < 0.01$). Using the definition of obesity as a total body fat greater than the 85th percentile of the range of the control groups (C-D and C-R), seven I-D chickens (64%) and eight I-R chickens (72%) were obese ($P < 0.02$ and < 0.004, respectively). Only four chickens (18%) were obese from the uninfected control groups together (C-D and C-R).

Serum lipids. Serum CHOL and TG for the I-D group and serum CHOL for the I-R group were significantly lower compared to the C-D group (Table 2).

Discussion

Whether Ad-36 causes viremia in the infected animals was previously unknown. Blood-borne spread of Ad-36 would be a possibility if Ad-36 does cause viremia. The aim of experiment 1 was limited to observing viral DNA titers in the blood. Therefore, only four animals were used for the experiment. The data from experiment 1 demonstrate that Ad-36 DNA appears in the blood after animals have been inoculated with Ad-36. The onset of Ad-36 DNA appearance in the blood was as soon as 12 h following the inoculation. Although the titers were gradually declining, Ad-36 DNA showed a prolonged and continued presence in the blood, which raises the possibility of a long carrier state for infected individuals. Substantially high titers for the viral DNA were present 36 h post-inoculation. Rise and fall of Ad-36 DNA titers in the blood suggested viral replication and clearance phases in the body. Appearance of Ad-36 DNA and the rapidity of the onset of viremia in the blood of chickens sharing cages with Ad-36 inoculated animals demonstrated the ease of transmission of Ad-36 infection in chickens. It appears that Ad-36 virus quickly spreads from the nasal passages of a chicken, circulates in the blood of the infected chickens, and is also very quickly spread horizontally to uninfected chickens kept in contact with the infected chickens. The exact route of infection of the 'in-contact' chickens is not clear. The results suggest a high degree of infectivity from either nasal–oral secretions and/or by fecal excretion and contamination. Both chickens in a cage were males and too young to be sexually active, which rules out sexual transmission of the infection.

Experiment 2 is the first report describing transmission of adiposity due to blood transfusions. We have previously shown that chickens and mice inoculated with Ad-36 develop adiposity and paradoxically low levels of serum CHOL and TG compared to uninfected controls.[8] In the present experiment, this adiposity syndrome was observed in the two infected groups. Relative to the total body fat increase, the infected chickens showed preferential increases in visceral adipose tissue. Presence of virus in the blood transfused to I-R chickens was demonstrated by isolating the virus and by DNA detection using CE. Also, the more than 4-fold rise in antibody titers, and the presence of Ad-36 DNA in adipose tissue and blood of the infected groups (I-D and I-R) confirm the presence of infection in these animals.

Selective presence of Ad-36 DNA in the visceral adipose tissue compared to the skeletal muscle despite its presence in the blood is an important finding and may indicate a viral affinity for the adipose tissue.

The results of the two experiments taken together suggest that Ad-36 can be transmitted from an experimentally infected chicken to a cage-mate through close physical contact, and that viral DNA may persist in blood for at least 3.5 weeks following acute infection. Not only the virus itself, but also the adiposity induced by the virus can be transmitted between chickens via blood.

One of the most significant findings of this project is the fulfilment of the Koch's postulate. Experiment 2 demonstrated that Ad-36 infection induced adiposity in animals. The same organism (Ad-36) could be recovered from the blood of these animals and on inoculation produced adiposity once again in a separate set of animals. These data along with our previous data that showed that adiposity induced by Ad-36 is not a non-specific effect of a viral infection[8] provide stronger evidence for a causative role for Ad-36 in inducing adiposity in animals under experimental conditions.

Ad-36 is a human virus and it was first isolated from a fecal sample of a diabetic girl with enteritis.[14] Almost nothing is known about its pathogenicity in humans. Serum neutralizing antibody assays show a widespread presence of Ad-36 antibodies in the population.[10] Therefore, Ad-36 pathogenicity has high relevance to human health. Obviously, we cannot demonstrate Ad-36 induced adiposity in humans by deliberate transfusion of blood contaminated with the virus. Therefore, we have used an animal model to study the transmissibility of Ad-36 infection and Ad-36 induced adiposity.

Conclusion

In conclusion, this study demonstrated that Ad-36 infection as well as Ad-36-induced adiposity could be transmitted in a chicken model. Further investigation is needed to investigate the potential for transmission of virus-induced adiposity in humans.

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Transmissibility of adenovirus-induced adiposity in a chicken model.

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BACKGROUND: We previously reported that human adenovirus Ad-36 induces adiposity and paradoxically lower levels of serum cholesterol (CHOL) and triglycerides (TG) in animals. OBJECTIVE: To evaluate the transmissibility of Ad-36 and Ad-36 induced adiposity using a chicken model. DESIGN: Experiment 1--four chickens were housed (two per cage) and one from each cage was inoculated with Ad-36. Duration of presence of Ad-36 DNA in the blood of all chickens was monitored. Experiment 2--two groups of chickens were intranasally inoculated with Ad-36 (infected donors, I-D) or media (control donors, C-D). Blood drawn 36 h later from I-D and C-D groups was inoculated into wing veins of recipient chickens (infected receivers, I-R, and control receivers, C-R, respectively). On sacrifice, 5 weeks post-inoculation, blood was drawn, body weight noted and visceral fat was separated and weighed. RESULTS: Experiment 1--Ad-36 DNA appeared in the blood of the inoculated chickens and that of uninoculated chickens (cage mates) within 12 h of inoculation and the viral DNA persisted up to 25 days in the blood. Experiment 2--compared with C-D, visceral and total body fat were significantly greater and CHOL significantly lower for the I-D and I-R. TG were significantly lower for the I-D. Ad-36 was isolated from 12 out of 16 blood samples of the I-D that were used for inoculating I-R chickens. Ad-36 DNA was present in the blood and the adipose tissue of the I-D and I-R but not in the skeletal muscles of animals selected randomly for testing. CONCLUSION: As seen in experiment 1, Ad-36 infection can be transmitted horizontally from an infected chicken to another chicken sharing the cage. Additionally, experiment 2 demonstrated blood-borne transmission of Ad-36-induced adiposity in chickens. Transmissibility of Ad-36-induced adiposity in chicken model raises serious concerns about such a possibility in humans that needs further investigation.

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Increased adiposity in animals due to a human virus.

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BACKGROUND: Four animal models of virus-induced obesity including adiposity induced by an avian adenovirus have been described previously. This is the first report of adiposity induced in animals by a human virus. OBJECTIVE: We investigated the adiposity promoting effect of a human adenovirus (Ad-36) in two different animal models. DESIGN: Due to the novel nature of the findings we replicated the experiments using a chicken model three times and a mammal model once. In four separate experiments, chickens and mice were inoculated with human adenovirus Ad-36. Weight matched groups inoculated with tissue culture media were used as non-infected controls in each experiment. Ad-36 inoculated and uninfected control groups were housed in separate rooms under biosafety level 2 or better containment. The first experiment included an additional weight matched group of chickens that was inoculated with CELO (chick embryo lethal orphan virus), an avian adenovirus. Food intakes and body weights were measured weekly. At the time of sacrifice blood was drawn and visceral fat was carefully separated and weighed. Total body fat was determined by chemical extraction of carcass fat. RESULTS: Animals inoculated with Ad-36 developed a syndrome of increased adipose tissue and paradoxically low levels of serum cholesterol and triglycerides. This syndrome was not seen in chickens inoculated with CELO virus. Sections of the brain and hypothalamus of Ad-36 inoculated animals did not show any overt histopathological changes. Ad-36 DNA could be detected in adipose tissue, but not skeletal muscles of randomly selected animals for as long as 16 weeks after Ad-36 inoculation. CONCLUSIONS: Data from these animal models suggest that the role of viral disease in the etiology of human obesity must be considered.

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TITLE: Determining if obesity in a person is caused by Ad-36 virus - and providing the basis for treatment or prevention of obesity-causing, cholesterol reducing adenovirus, using the purified variant, Ad-36p

INVENTOR: ATKINSON, R L; DHURANDHAR, N V

PRIORITY-DATA: 1997US-042942P (April 4, 1997), 1998US-0056153 (April 6, 1998)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
WO 9844946 A1	October 15, 1998	E	013	A61K039/235
AU 9868842 A	October 30, 1998		000	A61K039/235
US 6127113 A	October 3, 2000		000	C12Q001/70

INT-CL (IPC): A61 K 39/235; C12 N 7/00; C12 Q 1/68; C12 Q 1/70; G01 N 33/53

ABSTRACTED-PUB-NO: US 6127113A
BASIC-ABSTRACT:

A method to determine if a person is suffering viral obesity, comprising immunoassay or nucleic acid probe hybridisation of body fluid, faeces, or sample tissue, to detect infection by an obesity-causing and cholesterol reducing adenovirus, is new.Also claimed is substantially purified Ad-36p.

USE - The method is used to determine whether obesity in a person has a viral basis. Use of Ad-36p to detect viral infection and thus susceptibility to becoming obese, as the basis of a vaccine to prevent viral-based obesity, and as a method to reduce serum levels of total triglyceride, cholesterol, and low-density-lipoprotein-associated cholesterol, is disclosed.

ADVANTAGE - Substantially purified Ad-36p is more sensitive in immunoassays than the prior art ATCC Ad-36 culture, which contains a number of variants.

ABSTRACTED-PUB-NO:

WO 9844946A
EQUIVALENT-ABSTRACTS:

A method to determine if a person is suffering viral obesity, comprising immunoassay or nucleic acid probe hybridisation of body fluid, faeces, or sample tissue, to detect infection by an obesity-causing and cholesterol reducing adenovirus, is new.Also claimed is substantially purified Ad-36p.

USE - The method is used to determine whether obesity in a person has a viral basis. Use of Ad-36p to detect viral infection and thus susceptibility to becoming obese, as the basis of a vaccine to prevent viral-based obesity, and as a method to reduce serum levels of total triglyceride, cholesterol, and low-density-lipoprotein-associated cholesterol, is disclosed.

ADVANTAGE - Substantially purified Ad-36p is more sensitive in immunoassays than the prior art ATCC Ad-36 culture, which contains a number of variants.

ABSTRACTED-PUB-NO: US 6127113A
EQUIVALENT-ABSTRACTS: A method to determine if a person is suffering viral obesity, comprising immunoassay or nucleic acid probe hybridisation of body fluid, faeces, or sample tissue, to detect infection by an obesity-causing and cholesterol reducing adenovirus, is new.Also claimed is substantially purified Ad-36p. USE - The method is used to determine whether obesity in a person has a viral basis. Use of Ad-36p to detect viral infection and thus susceptibility to becoming obese, as the basis of a vaccine to prevent viral-based obesity, and as a method to reduce serum levels of total triglyceride, cholesterol, and low-density-lipoprotein-associated cholesterol, is disclosed. ADVANTAGE - Substantially purified Ad-36p is more sensitive in immunoassays than the prior art ATCC Ad-36 culture, which contains a number of variants. WO 9844946A

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PAPER

Human adenovirus-36 is associated with increased body weight and paradoxical reduction of serum lipids

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BACKGROUND: Human adenovirus-36 (Ad-36) increases adiposity and paradoxically lowers serum cholesterol and triglycerides in chickens, mice, and non-human primates. The role of Ad-36 in human obesity is unknown.
OBJECTIVES: To determine the prevalence of Ad-36 antibodies in obese and nonobese humans. To evaluate the association of Ad-36 antibodies with body mass index (BMI) and serum lipids.
DESIGN: Cohort study. Volunteers from obesity treatment programs, communities, and a research study.
SUBJECTS: Obese and nonobese volunteers at the University of Wisconsin, Madison, WI, and the Bowen Center, Naples, Florida. Obese and thin volunteer research subjects and 89 twin pairs at Columbia University, New York.
INTERVENTIONS: Study 1: 502 subjects; serum neutralization assay for antibodies to Ad-2, Ad-31, Ad-36, and Ad-37; serum cholesterol and triglycerides assays. Study 2: BMI and %body fat in 28 twin pairs discordant for Ad-36 antibodies.
MAIN OUTCOME MEASURES: Presence of antibodies to adenoviruses, BMI, serum cholesterol and triglycerides levels.
RESULTS: Significant ($P<0.001$) association of obesity and positive Ad-36 antibody status, independent of age, sex, and collection site. Ad-36 antibodies in 30% of obese, 11% of nonobese. Lower serum cholesterol and triglycerides ($P<0.003$) in Ad-36 antibody-positive *vs* -negative subjects. Twin pairs: antibody-positive twins had higher BMIs (24.5 ± 5.2 *vs* 23.1 ± 4.5 kg/m^2, $P<0.03$) and %body fat ($29.6\pm9.5\%$ *vs* $27.5\pm9.9\%$, $P<0.04$). No association of Ad-2, Ad-31, or Ad-37 antibodies with BMI or serum lipids.
CONCLUSIONS: Ad-36 is associated with increased body weight and lower serum lipids in humans. Prospective studies are indicated to determine if Ad-36 plays a role in the etiology of human obesity.
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Keywords: body mass index; etiology; serum cholesterol; serum triglycerides; twins; viral antibodies

Introduction

Four animal viruses and the scrapie agent are reported to increase adiposity when injected into animals.[1–11] Canine distemper virus produces obesity in mice, probably due to viral-induced hypothalamic damage.[1–5] Rous-associated virus type 7 (RAV-7) induces a syndrome of obesity, stunted growth, hyperlipidemia, fatty liver, hepatomegaly, anemia, and immune suppression in chickens.[6,7] Borna virus produces an obesity syndrome in rats characterized by lymphomonocytic inflammation of the hypothalamus, pancreatic islet hyperplasia, and elevated serum glucose and triglycerides levels.[8]

Dhurandhar *et al*[10,11] reported that an avian adenovirus, SMAM-1, produced excessive deposition of visceral fat and paradoxical reductions of serum cholesterol and triglycerides in chickens. Of 52 obese humans tested, 10 had antibodies to SMAM-1. These individuals had a higher body mass index (BMI) and lower serum cholesterol and triglycerides than did antibody-negative subjects.[12]

Our laboratory reported that inoculation of a human adenovirus-36, (Ad-36), into chickens, mice, and non-human primates produces a distinctive syndrome of increased adipose tissue with paradoxically lower serum cholesterol and triglycerides compared to uninfected controls.[13–15]

The current study evaluated the association of positive antibody status to four human adenoviruses, Ad-36, Ad-2, Ad-31, and Ad-37, with body weight and serum lipids in

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obese and nonobese humans and in twin pairs discordant for Ad-36 antibodies.

Methods

We performed two studies. In Study 1, serum from obese and nonobese volunteers in three American cities was assayed for viral antibodies, cholesterol, and triglycerides. In Study 2, we assayed samples from twin pairs. Written informed consent was obtained for collection of blood samples. The University of Wisconsin Human Subjects Committee approved the protocols.

Subjects, Study 1—Obese and nonobese volunteers

Blood samples were obtained from 502 subjects from Madison, Wisconsin (WI); Naples, Florida (FL); and New York, (NY) (Table 1). Overweight was defined as a BMI of $\geq 25\,kg/m^2$, and obesity was defined as a BMI of $\geq 30\,kg/m^2$.[16] Overweight and obese volunteers in Madison and Naples were attending weight reduction programs. Blood was obtained at or near the time of entry into the program in most subjects. Nonobese subjects were volunteers from the University and local communities obtained by word-of-mouth publicity during the period from 1995 to 1999. Aliquots of stored samples were obtained from obese and thin research subjects at the New York Obesity Research Center, who were massively obese (lifetime BMI $\geq 50\,kg/m^2$) or unusually thin (BMI $\leq 22\,kg/m^2$) during a period from 1993 to 1998. Race was not specifically ascertained, but of the Madison and Naples subjects, more than 95% were of Caucasian ancestry. In New York, about one-third were black subjects and about one-half were Caucasian.

Subjects, Study 2—twin samples

Serum samples from 89 twin pairs, collected from about 1993 to 1998 for other studies, were screened for antibodies to Ad-36. A total of 26 twin pairs who were discordant for Ad-36 antibody status (20 monozygotic; six dizygotic) had assessments of BMI, cholesterol, and triglyceride levels. Each subject had percent body fat (% body fat) measured using dual-energy X-ray absorptiometry, hydrodensitometry, and/or bioimpedance analysis. The same body composition method(s) was used within twin pairs.

Development of assays for human adenoviruses

Table 1 Characteristics of human subjects, Study 1

	Florida		*New York*		*Wisconsin*	
	Obese	*Nonobese*	*Obese*	*Nonobese*	*Obese*	*Nonobese*
Number	103	35	74	43	183	64
Men/women	21/82	7/28	19/55	1/42	47/136	17/47
Age (y)	45.3	42.9	38.5	29.7*	44.2	35.1*
(±s.d.)	(±10.6)	(±13.7)	(12.6)	(±7.2)	(±8.2)	(±11.2)
BMI (kg/m^2)	37.4	26.9	61.2	20.3	39.8	25.3
(±s.d.)	(±6.9)	(±2.8)	(±12.0)	(±2.2)	(±8.2)	(±3.3)

Definition of obesity: BMI $\geq 30\,kg/m^2$; Mean ± standard deviation. *$P<0.001$.

Human adenoviruses types 2 (Ad-2), 31 (Ad-31), 36 (Ad-36), and 37 (Ad-37) from the American Type Culture Collection (ATCC, Manassas, VA, USA) were grown on A549 cells, a human bronchial carcinoma tissue culture line. TCID-50, the dose of virus that produced cell damage (CPE) in 50% of wells, was determined for each virus by standard methods.[13] A sample of Ad-36 virus from a single plaque on a tissue culture plate was plated onto fresh cells and the process repeated three times to obtain a homogenous virus work stock.[13] Use of this purified Ad-36 virus stock (Ad-36P) increased sensitivity of antibody detection in Ad-36-positive serum samples compared to the original Ad-36 source, so Ad-36P was used in serum neutralization (SN) assays for all but the first 105 samples from Wisconsin, and for all assays from the other sites.

SN assays for viral antibodies in serum were performed using the 'constant virus-decreasing serum' method.[13] Test serum was heat inactivated in a water bath at 56°C for 30 min and serially diluted (two-fold) from 1:2 to 1:512 in 96-well plates. A total of 100 TCID-50 of the respective adenovirus work stock was added to each of the wells. A549 cells were added to these wells after 1 h of incubation at 37°C. Each test serum was run in duplicate by individuals blinded to the subjects' BMI. Serum control (serum and cells, but no virus), cell control (cells alone, no virus, no serum), and virus control (cells and virus, no serum) were included with each assay. Plates were incubated at 37°C for 11 days and the presence of CPE was noted. Serum samples without CPE in dilutions of 1:8 or higher were considered positive for the presence of neutralizing antibodies to the respective virus and evidence of prior infection with that virus. Samples with titers lower than 1:8 were considered nonspecific in the SN assay.

A virus back-titration was carried out with each assay as a quality check. In all, 100 TCID-50 of the virus was added to the first well of the 96-well plate and serial two-fold dilutions were made. A549 cells were added to the wells and development of CPE was observed. The amount of virus decreases with each dilution, and infectivity was never observed with seven or more dilutions.

Assays for serum cholesterol and triglycerides

Serum samples from Wisconsin subjects were assayed for cholesterol and triglycerides by the University of Wisconsin Hospital Clinical Laboratory. Florida and New York serum samples were assayed using cholesterol oxidase–peroxidase colorimetric Sigma kit assays (Sigma, St. Louis, cat #352) with a cholesterol calibrator (Sigma, cat #C 7921) and cardiolipid control (Sigma, cat #C 4571). Subjects from Florida and New York were not necessarily fasting, so triglyceride assays were not performed on their serum.

Statistical analyses

Study 1: Ad-36 antibodies in three sites

To test the overall effect of the presence of Ad-36 antibodies on the presence of obesity, we regressed BMI

status on Ad-36 antibody status while controlling for age, sex, and site of recruitment (Florida, New York, Wisconsin) using logistic regression. It was not appropriate to use BMI as a continuous variable in a linear regression analysis because the nature of the nonrandom extreme sampling on the BMI phenotype that was employed at each site led to marked violations of the parametric assumptions (ie, normality of residuals and homoscedasticity) of such an analysis. To test the sensitivity of our results to different choices of cutpoint, we repeated the analysis several times re-setting the BMI cutpoint for each integer value from 25 to 30 inclusive. Regardless of which BMI cutpoint was chosen, results were unchanged in terms of both direction of effect and statistical significance. The cutpoint that gave the largest χ^2 (or Akaike's Information Criterion—AIC) value in the logistic regression was 27 kg/m^2. However, we chose to present the data based on the WHO and NIH definition of obesity ($\geq$30 kg/m^2) as the reference value. Data from all three sites were pooled in a single analysis and potential differences in effects among sites were tested via using interaction terms between two dummy codes for site and Ad-36 status. Data were also analyzed in a secondary analysis separately by site.

To test the effect of Ad-36 on susceptibility to lower serum cholesterol levels, we regressed cholesterol level on Ad-36 antibody status while controlling for age, sex, site of recruitment, and BMI (to control for the greater obesity among the Ad-36-positive subjects) using ordinary least-squares linear regression. To improve the fit of the data to the parametric assumptions of the analysis, cholesterol was transformed via a Box-Cox transformation[17] by raising cholesterol levels to the power of 0.25. The results were virtually identical when the untransformed data were analyzed. As above, data from all three sites were pooled in a single analysis and potential differences in effects among sites were tested via using interaction terms and then analyzed in a secondary analysis separately by site.

Fasting serum for triglycerides assays were available only from Wisconsin, so site-specific analyses were not performed. Data analyses, using a Box-Cox transformation and raising triglycerides levels to the power of 0.25, were similar to those for cholesterol. The results were virtually identical when the untransformed data were analyzed.

Study 2: twins discordant for Ad-36 antibodies

A weighted least-squares analysis was applied to the monozygotic and dizygotic twin pairs, followed by *t*-tests to test for intrapair differences in BMI, serum cholesterol, and triglycerides.

Results

Study 1, Comparison of obese and nonobese subjects

Table 1 shows that women outnumbered men in all three sites and that nonobese subjects were younger than obese subjects ($P<0.001$) in two sites. However, the mean age was similar for Ad-36 antibody-positive (AB+) and antibody-negative (AB−) subjects (age = 41.7 *vs* 40.7 y, $P=0.38$) (Table 2), suggesting that age did not play a role in susceptibility to infection with Ad-36.

Table 2 Ad-36 antibody status, age, and BMI in obese and nonobese subjects

	Age (y)	*BMI (kg/m^2)*
All subjects combined		
AB− ($N=378$)	40.7±11.6	35.8±12.3
AB+ ($N=124$)	41.7±10.3	44.9±16.3*
Nonobese (BMI<30)		
AB− ($N=126$)	35.1±12.0	23.9±3.8
AB+ ($N=16$)	36.1±9.0	26.3±4.2**
Obese (BMI≥30)		
AB− ($N=252$)	43.6±10.2	41.8±10.6
AB+ ($N=108$)	42.5±10.3	47.7±15.6*

Mean±s.d.; *$P<0.0001$; **$P<0.02$. AB−: Ad-36 antibody negative; AB+: Ad-36 antibody positive.

Results of testing for Ad-36 antibodies

Percent antibody positive: When obese subjects from all sites were combined, 30% were Ad-36 AB+ compared to 11% of all the nonobese subjects. The percent of Ad-36 AB+ obese individuals was 27% in Florida, 58% in New York, and 20% in Wisconsin, and the percentages for Ad-36 AB+ nonobese individuals were 14, 9, and 11%, respectively.

Body mass index: In the overall analysis, independent of age and sex, the effect of Ad-36 status on the risk of obesity was highly significant ($\chi^2=34.0$, df = 1; $P<0.001$). Moreover, the 2 df interaction test of whether the results differed by site was not significant ($\chi^2=0.618$, df = 2; $P=0.74$) indicating that the results were consistent across sites. Similarly, when data were analyzed separately by site, the estimated effect of Ad-36 remained significant (FLA, $\chi^2=5.99$, df = 1; $P<0.02$; NY, $\chi^2=21.19$, df = 1; $P<0.001$; WI, $\chi^2=8.34$, df = 1; $P<0.005$).

With all subjects combined, the mean BMI of Ad-36 AB+ subjects was 44.9±16.3 kg/m^2 *vs* a BMI of 35.8±12.3 kg/m^2 in AB− subjects (Table 2). In nonobese subjects, positive Ad-36 antibody status was associated with a modestly greater BMI (26.3±4.2 *vs* 23.9±3.8 kg/m^2). Obese AB+ subjects had a BMI of 47.7±15.6 *vs* 41.8±10.6 kg/m^2 for the AB− subjects (Table 2).

Serum cholesterol and triglycerides: As we previously observed in three species of animals infected with Ad-36, both obese and nonobese subjects who were Ad-36 AB+ had paradoxically lower levels of cholesterol and triglycerides, which were highly significantly different from individuals who were Ad-36 AB− (Table 3). With all subjects combined, serum cholesterols were 5.51±0.07 in AB− *vs* 4.64±0.11 mmol/l in AB+ ($P<0.0001$). When data were analyzed separately by site, the estimated effect remained significant (Table 3). Fasting serum triglycerides were

Table 3 Ad-36 antibody status and serum lipids

	Cholesterol (mmol/l)		*Triglycerides (mmol/l)*	
	AB–	*AB+*	*AB–*	*AB+*
Florida	5.47±0.15	4.53±0.25*	—	—
New York	5.30±0.19	4.41±0.21*	—	—
Wisconsin	5.60±0.09	4.97±0.12*	1.70±0.07	1.29±0.07**
Combined sites	5.51±0.07	4.64±0.11**	1.70±0.07	1.29±0.07**

Mean±s.e.m.; *$P<0.003$; **$P<0.0001$. AB–: Ad-36 antibody negative; AB+: Ad-36 antibody positive.

available only at the Wisconsin site. In 108 fasting subjects, triglycerides were 1.70±0.07 in AB– and 1.29±0.07 mmol/l ($P<0.0001$) in AB+ (Table 3).

Examining the data in a different manner by focusing on serum cholesterols, the subjects were divided into quartiles based on serum cholesterol. The lowest quartile of cholesterol had 42% of subjects AB+ for Ad-36 whereas the top quartile had 11% of subjects AB+. As expected, age increased from the bottom to the top quartile (38.1±11.5 *vs* 43.4±11.4, $P<0.0007$), but BMI was higher in the bottom quartile (41.6±15.6 *vs* 36.7±13.1, $P<0.008$).

Other variables: A total of 31 other variables were evaluated for the first 106 samples from obese subjects in Wisconsin, including waist and hip circumferences, smoking status, blood pressure, pulse rate, high-density lipoproteins (HDL), glucose, uric acid, thyroid function tests (T4, TSH), and other standard lab tests. The age of onset of obesity trended towards significance in subjects positive *vs* negative for Ad-36 antibodies (17.9±2.6 *vs* 13.0±1.2 y (±s.e.m.), $P<0.10$). None of the other variables was significantly correlated with antibody status.

Results of testing for Ad-2, Ad-31, and Ad-37

Body weight: In subjects with sufficient sera remaining for the assay, SN assays were performed for antibodies to Ad-2, Ad-31, and Ad-37. The prevalence of antibodies in obese *vs* nonobese was not different for Ad-2 (76 *vs* 81%, $P=0.4$) or for Ad-31 (70 *vs* 80%, $P=0.2$) (Table 4). One obese and four nonobese subjects out of a total of 198 subjects screened had antibodies to Ad-37. These five subjects were not analyzed further. Combining obese and nonobese subjects, analyses revealed no significant differences for age, BMI, or triglycerides between the AB+ and AB– subjects for either Ad-2 or Ad-31.

Serum cholesterol and triglycerides: No significant associations of serum cholesterol or triglycerides were noted with Ad-2 or Ad-31 antibody status, with the exception of a significantly lower ($P<0.05$) serum cholesterol in nonobese subjects positive for Ad-2 antibodies (Table 4). These results suggest that the increased prevalence of adenovirus antibodies in obese subjects and the paradoxical lowering of serum cholesterol and triglycerides associated with the presence of Ad-36 antibodies is not common to all human adenoviruses.

Table 4 Antibody status of Ad-2 and Ad-31 and characteristics of obese and nonobese subjects

	Obese		*Nonobese*	
	Antibody+	*Antibody–*	*Antibody+*	*Antibody–*
Ad-2				
Number	111	34	42	10
Age (y)	46.0±9.9	44.0±12.0	30.9±9.7	36.2±18.1
BMI (kg/m^2)	39.1±9.0	38.2±7.0	22.2±2.5	21.8±1.6
Cholesterol (mmol/l)	5.37±1.20	4.95±1.46	5.24±1.48	7.17±2.29*
Ad-31				
Number	107	45	39	10
Age (y)	45.8±9.9	44.3±13.9	31.9±12.1	30.5±12.1
BMI (kg/m^2)	37.6±8.4	35.3±5.7	21.6±2.1	23.5±1.9*
Cholesterol (mmol/l)	5.02±1.28	5.35±1.40	5.50±1.87	6.08±1.84

Mean±standard deviation. *$P<0.05$.

Study 2, twin studies

Studies of Ad-36: Of the 178 twins 40 (22%) had antibodies to Ad-36. Six pairs were concordant Ad-36 AB+, 56 pairs were concordant AB–, and 28 pairs were discordant for Ad-36 antibodies. In the 28 twin pairs who were discordant for Ad-36 antibodies, the individuals who were positive for Ad-36 antibodies were heavier and fatter than their co-twins who did not have Ad-36 antibodies. There were no differences in serum lipids in the discordant twin pairs (Table 5). The specific analyses were as follows:

For both BMI and % body fat, there was a clear effect of Ad-36 status. AB+ twins had a BMI of 26.1 *vs* 24.5 kg/m^2 ($P<0.04$) and % body fat was 29.6 *vs* 27.5 ($P<0.04$). There were no differences between AB+ and AB– for cholesterol and triglycerides.

Studies of Ad-2, Ad-31, and Ad-37: In contrast to Ad-36, there was no association of BMI and antibodies to Ad-2, Ad-31, and Ad-37 in twins discordant for antibodies to these viruses. The BMIs of AB+ *vs* AB– co-twins were 24.8±4.1 kg/m^2 (±s.d.) *vs* 24.0±2.9 kg/m^2 ($P=0.21$, $N=8$) for Ad-2, 24.1±4.9 *vs* 25.3±6.1 kg/m^2 ($P=0.08$, $N=23$) for Ad-31, and 28.1±10.2 *vs* 26.5±7.2 kg/m^2 ($P=0.36$, $N=8$) for Ad-37. There were no significant differences in serum cholesterol or triglycerides based on antibody status for any of the three viruses.

Discussion

Animal data show that inoculation of Ad-36 reproducibly produces a syndrome of increased visceral adipose tissue and reduced serum cholesterol and triglycerides in chickens, mice, and non-human primates.[13–15] Infection of human subjects cannot be performed for ethical reasons, so direct proof that Ad-36 increases body fat and lowers serum lipids in humans will be difficult to obtain. This study demon-

Table 5 Characteristics of twin sample ($n=28$ pairs), Study 2

	Antibody positive	*Antibody negative*
Age (y)	33.0±15.7	33.0±15.7
Sex (% female/male)	79/21	79/21
BMI (kg/m^2)	26.1±9.8	24.5±9.5*
Body fat (%)	29.6±9.5	27.5±9.9*
Cholesterol (mmol/l)	4.73±1.37	4.75±1.26
Triglycerides (mmol/l)[a]	1.48±0.59	1.48±0.42

±Standard deviation. *$P<0.04$ *vs* antibody positive. [a]Triglycerides measured on only 16 of the twin pairs because the others were not fasting.

strated that both obese and nonobese subjects positive for Ad-36 antibodies had significantly higher BMIs and lower serum cholesterol and triglycerides levels compared to AB− subjects, thus demonstrating a syndrome similar to animals infected with Ad-36 in the laboratory. These data show an association, but causality for human obesity has not been proven.

Our data show that the prevalence of Ad-36 antibodies is almost three-fold higher in obese *vs* nonobese individuals. To explain this, we considered the possibility that obese people might be more susceptible to Ad-36 infection than nonobese. Our data on antibody status for Ad-2 and Ad-31 offer the most compelling evidence that obese people are not more susceptible to infection. We found that the prevalence of antibodies to Ad-2 and Ad-31 was similar between obese and nonobese, and that these human adenoviruses were not associated with changes in adiposity or serum lipids. Other evidence to refute this hypothesis comes from the literature on antibody formation in obese people. Exposure to Ad-36 would be expected to produce antibodies in both nonobese and obese people. However, there are data suggesting that obese people have impaired immune function. Obese people vaccinated with hepatitis B virus have a reduced prevalence of antibodies, suggesting that immune response to this viral antigen is decreased.[18] This finding might suggest that obese people would be less likely to produce antibodies when exposed to Ad-36 than would lean people. It is unlikely that obese people would be exposed to Ad-36 more often than lean people. Thus, the prevalence of Ad-36 antibodies would be expected to be similar or lower in obese people, not higher.

The average age of obese subjects was greater than that of nonobese, but the average age of AB+ subjects was similar among obese and nonobese. Thus, it seems unlikely that an increased chance of viral infection simply due to age could explain the differences in Ad-36 antibody status between obese and nonobese.

Evidence for an association of Ad-36 with increased body weight and adiposity in humans is provided by the twin data in this study. Within pairs, twins normally have a close correlation of body weight and body fat. Bouchard and coworkers[19–22] demonstrated a higher correlation of body fat and visceral fat within twin pairs, compared to among twin pairs, with or without dietary perturbations. It is possible that there may be other confounding variables not considered, but our findings of a significantly higher BMI and % body fat in the twins with Ad-36 antibodies support the hypothesis that Ad-36 increases adiposity in humans. We cannot explain the lack of a difference in serum lipids in discordant twins pairs, but most pairs were not obese and the length of time from exposure to the virus to our measurements could not be calculated.

Ad-36 appears to differ from other human adenoviruses. There is no immunological crossreactivity between Ad-36 and 47 of the 49 other human adenoviruses in the ATCC.[23,24] The other human adenoviruses tested in this study, Ad-2, Ad-31, and Ad-37, did not have differences in antibody status between obese and nonobese. Twins discordant for antibodies to these other viruses had no differences in body weight or body fat. Serum cholesterol was lower in nonobese humans positive for Ad-2 antibodies, but levels of serum lipids were not otherwise correlated with presence of antibodies to Ad-2, Ad-31, or Ad-37. It appears that increases in adiposity and reduction of serum lipids are not routine consequences of infection with human adenoviruses.

The mechanisms by which Ad-36 increases adiposity in animals are not yet known. At least two other animal viruses produce obesity in animal models by damaging the hypothalamus. Our preliminary studies in animals did not demonstrate any lesions of the hypothalamus or other areas of the brain.[13] In preliminary studies, we have demonstrated that DNA of Ad-36 may be isolated from adipose tissue of chickens 13 weeks after inoculation, non-human primates after 28 weeks, and in human adipose tissue from autopsies.[25,26] Also, *in vitro* studies in the 3T3-L1 tissue culture adipoblast cell line suggest that Ad-36 may alter adipocyte differentiation.[25] As compared to control cells, cells infected with Ad-36 differentiate much faster and accumulate triglycerides more rapidly. A control virus, Ad-2, did not have this effect. We postulate that Ad-36 affects fat cells directly, leading to an increased fat cell number and increased fat cell size.[25]

The mechanisms of alterations in serum lipids in both obese and nonobese humans also are unknown. Acute viral infections may lower serum cholesterol, but often produce elevations in serum triglycerides. The other animal models of virus-induced obesity are associated with elevated triglycerides.[1–8] Finally, it is not clear if the associations of elevated BMI and lowered serum lipids with positive Ad-36 antibody status are linked, or are associated by different mechanisms. None of the other adenoviruses tested had any associations with either BMI or serum lipids, with the exception of Ad-2. Serum cholesterol was higher in the nonobese subjects AB+ for Ad-2, but there were only 10 people in this group and the standard deviation was high, so this may be a chance observation.

Ad-36 was first isolated in 1978 in a diabetic child in Germany.[23] More research is needed to determine if Ad-36

has played any role in the global epidemic of obesity that has occurred over the last approximately 20 y.[16,27]

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